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Abnormalities in cortical interneuron subtypes in ephrin-B mutant mice

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Abstract

To explore roles for ephrin-B/EphB signaling in cortical interneurons we previously generated *ephrin-B* (*Efnb1/b2/b3*) conditional triple mutant (TM^{flz}) mice using a *Dlx1/2*.Cre inhibitory neuron driver and green fluorescent protein (GFP) reporters for the two main inhibitory interneuron groups distinguished by expression of either glutamic acid decarboxylase 1 (GAD1; GAD67-GFP) or 2 (GAD2; GAD65-GFP). This work showed a general involvement of ephrin-B in migration and population of interneurons into the embryonic neocortex. We now determined if specific interneurons are selectively affected in the adult brains of TM^{flz} .Cre mice by immunostaining with antibodies that identify the different subtypes. The results indicate that GAD67-GFP expressing interneurons that also express parvalbumin (PV), calretinin (CR), and to a lesser extent somatostatin (SST) and Reelin (*Rln*) were significantly reduced in the cortex and hippocampal CA1 region in TM^{flz} .Cre mutant mice. Neuropeptide Y (NPY) interneurons that also express GAD67-GFP were reduced in the hippocampal CA1 region, but much less so in the cortex, though these cells exhibited abnormal cortical layering. In GAD65-GFP expressing interneurons, CR subtypes were reduced in both cortex and hippocampal CA1 region, whereas *Rln* interneurons were reduced exclusively in hippocampus, and the numbers of NPY and vasoactive intestinal polypeptide (VIP) subtypes appeared normal. PV and CR subtype interneurons in TM^{flz} .Cre mice also exhibited reductions in their perisomatic area, suggesting abnormalities in dendritic/axonal complexity. Altogether, our data indicate that ephrin-B expression within forebrain interneurons is required in specific subtypes for their normal population, cortical layering, and elaboration of cell processes.

Graphical Abstract

Many different interneuron subtypes in the cortex and hippocampus are altered in ephrin-B inhibitory neuron mutant mice. We utilized tdTomato fluorescence to identify inhibitory neurons exposed to Cre recombinase (red), GFP fluorescence to identify either GAD67 or GAD65

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expressing inhibitory neurons (green), and antibodies to identify specific interneuron subtypes (purple). Shown is a confocal image of merged tdTomato, GAD67-GFP, and somatostatin signals identifying interneurons in the cortex.

Keywords

Interneuron subtypes; Eph-Ephrin bidirectional signaling; cortex; hippocampus; CA1

Introduction

GABAergic inhibitory neurons are a fundamental component of the nervous system that together with excitatory neurons and glia form properly balanced neural circuits that are crucial for normal brain development and function. Inhibitory interneurons comprise approximately 20–30% of the cortical neurons (Gelman and Marin, 2010; Markram et al., 2004; Sultan et al., 2013) and exhibit a rich diversity of subtypes based on molecular markers (see below), morphology, and electrophysiology (Faux et al., 2012; Gelman and Marin, 2010; Markram et al., 2004; Sultan et al., 2013). These interneurons are born in subpallial ganglionic eminences (GEs) of the developing embryonic forebrain and migrate in a tangential fashion to populate the cortex and hippocampus (Faux et al., 2012; Gelman and Marin, 2010; Sultan et al., 2013). While inhibitory interneurons that migrate into the cortex/hippocampus are derived from *Dlx1/2*-expressing progenitor cells, they can be subdivided into three main groups based on their GE birth origin. Approximately 70% of cortical inhibitory interneurons are derived from the medial ganglionic eminence (MGE) while ~30% are derived from the caudal ganglionic eminence (CGE) (Faux et al., 2012; Gelman and Marin, 2010; Sultan et al., 2013). A small portion of cortical interneurons (<5%) further originate from the preoptic area (POA) (Gelman and Marin, 2010; Sultan et al., 2013). It has been shown that at least a proportion of MGE-derived interneurons express GAD1/GAD67 (Lopez-Bendito et al., 2004; Tamamaki et al., 2003) while majority of CGE-derived express GAD2/GAD65 (Lopez-Bendito et al., 2004). MGE derived cells can be further divided to

two subpopulations, Parvalbumin (PV; ~40% of cortical interneurons) expressing cells predominantly located in cortical layers V/VI and Somatostatin (SST; ~30%) expressing cells more abundant in cortical layer V. PV expressing interneurons show either multipolar basket cell or chandelier cell morphology, while majority of SST expressing interneurons exhibit a so-called Martinotti cell morphology. Some SST expressing cells can also express the calcium binding protein Calretinin (CR), the secreted glycoprotein Reelin (Rln), or Neuropeptide Y (NPY). On the other hand, CGE-derived interneurons mainly exhibit bipolar cell or double bouquet cell morphology and are distributed across all layers, predominantly in layer I and II. As in the MGE derived group, some CGE derived cells also express CR more abundant in cortical layers II/III, Rln, or NPY. Further, some CGE-derived interneurons express exclusively the neuropeptide vasoactive intestinal polypeptide (VIP) (Faux et al., 2012; Gelman and Marin, 2010; Markram et al., 2004; Sultan et al., 2013). GFP reporter mice have been generated for both GAD67 and GAD65 that faithfully label their respective interneuron subtypes. As such, GAD67-GFP has been shown to brightly label PV, SST, CR, NPY, and Rln subtypes while GAD65-GFP labels other subtypes of CR, NPY, and Rln, as well as VIP (Fukuda et al., 1997; Lopez-Bendito et al., 2004; Pesold et al., 1998; Tamamaki et al., 2003; Wierenga et al., 2010).

We previously identified a cell autonomous role for the three transmembrane ephrin-B proteins in the tangential migration of cortical/hippocampal interneurons using *ephrin-B* (*EfnB1/B2/B3*) conditional triple mutant ($TM^{Δz}$) mice and an inhibitory neuron specific *Dlx1/2.Cre* driver (Talebian et al., 2017). We found that the numbers of both GAD67-GFP and GAD65-GFP expressing interneurons that migrated into the cortex and hippocampus were strongly reduced in *Dlx1/2.Cre* containing $TM^{Δz}$ mice ($TM^{Δz}.Cre$). While *Dlx1/2.Cre*-mediated loss of ephrin-B does not impact the absolute number of inhibitory neurons in the embryonic forebrain (which includes cortical/hippocampal interneurons and other inhibitory neurons in the developing striatum) or affect changes in their proliferation, death, or fate/differentiation into GAD67 and GAD65 groups, we found the interneurons within the cortex exhibited defects in the leading process involved in tangential migration which was associated with their accumulating in lateral regions of the adult brain. However, it was not determined whether all interneuron subtypes are affected by cell autonomous loss of ephrin-B or if specific subtypes are affected more than others. Thus, using the same conditional strategy, we now investigated the distribution and morphology of interneuron subtype in the somatosensory cortex and CA1 region of the hippocampus.

Materials and Methods

Experimental Design

The conditional *ephrin-B* triple mutant ($TM^{Δz}$) mice containing the *Ai9*tdTomato (Tom) indicator of Cre activity as well as GAD67-GFP or GAD65-GFP reporters were generated as previously described (Talebian et al., 2017). Brains from *Dlx1/2.Cre* containing $TM^{Δz}$ and wild-type mice ($TM^{Δz}.Cre$ and WT.Cre) that also carried either GAD67-GFP or GAD65-GFP reporters were fixed, vibratome sectioned, immunostained using specific antibodies against PV, SST, CR, Rln, NPY, and VIP subtypes, treated with appropriate Alexa 647-conjugated secondary antibodies, and then imaged using a confocal microscope for Cy5,

Tom, GFP, and DAPI fluorescence. The total number of Cy5⁺ subtype-specific neurons were counted and quantified in the somatosensory cortex and hippocampal CA1 region, including those that were exposed to Dlx1/2.Cre recombinase and expressed a GAD-GFP (Cy5⁺/Tom⁺/GFP⁺ triple-positive cells), those that were exposed to Dlx1/2.Cre regardless of any GAD-GFP expression (Cy5⁺/Tom⁺), those that expressed GAD-GFP regardless of whether they were exposed to Dlx1/2.Cre or not (Cy5⁺/GFP⁺), and those that only expressed the subtype-specific marker and were not exposed to Dlx1/2.Cre recombinase and did not express GFP (Cy5⁺/Tom⁻/GFP⁻). Differences between *TM^{flz}*.Cre and WT.Cre brains were statistically analyzed.

Mice

All mice used in this study have been previously described; GAD65-GFP (Lopez-Bendito et al., 2004) and GAD67-GFP (Tamamaki et al., 2003) reporters, loxP flanked alleles in *ephrin-B1* (*EfnB1^{loxP}*) (Davy et al., 2004), *ephrin-B2* (*EfnB2^{loxP}*) (Gerety and Anderson, 2002), and *ephrin-B3* (*EfnB3^{flz}*) (Yokoyama et al., 2001), Dlx1/2.Cre driver (Potter et al., 2009), and Rosa26-STOP-tdTomato Cre indicator (*Ai9*) (Madisen et al., 2010). Mice were maintained in a mixed CD1/129 genetic background. Experiments were carried out using both female and male mice in accordance with the US National Institutes of Health Guide for the Care and Use of Animals under an Institutional Animal Care and Use Committee approved protocol and at an Association for Assessment and Accreditation of Laboratory Animal Care approved facility at the University of Texas Southwestern Medical Center. Animals were generated by M.H. and brain tissues were collected, fixed, genotyped, and provided to A.T. and R.B. for blinded analysis.

Brains slice preparation

Adult mouse brains were collected around postnatal day 90 (P90) from separate groups of mice containing either GAD67-GFP or GAD65-GFP and from either WT.Cre or *TM^{flz}*.Cre. Mice were anesthetized using 225 mg/kg ketamine and 25 mg/kg xylazine mixture in PBS and perfused with PBS followed by 4% paraformaldehyde in PBS. Brains were collected and post-fixed overnight in the dark in 4% paraformaldehyde in PBS, washed multiple times with PBS, and then stored in PBS containing 0.05% sodium azide in the dark at 4°C. Fixed brains were embedded in 3% agarose and 50 µm coronal sections were cut with a vibratome (frequency 7 Hz, speed 5 Hz). Slices were selected between interval 2.36 mm –1.64 mm (Bregma –1.43 to –2.15 mm) with approximately 700 µm thickness giving around 12–15 slices per brain. Slices were placed in 24-well plates (1–2 slices in each) in PBS containing 0.05% sodium azide and maintained in the dark at 4°C until subjected to antibody labeling.

Immunofluorescence

Free floating brain sections were blocked in 24-well plates in blocking solution (8% donkey serum, 4% bovine serum albumin, 0.1% Triton X-100 in PBS) for 1 hour at RT and then probed with primary antibodies diluted in blocking solution overnight (CR, PV) or 48 h (SST, NPY, Rln, VIP) at 4°C. Sections were then washed 3 × 30 min in PBST (PBS containing 0.1% Tween-20) and probed with Alexa 647-conjugated secondary antibodies (1:500; Jackson ImmunoResearch) for 1 hour at RT with DAPI (0.02 mg/ml; Sigma #D9542). Sections were then washed in PBST 3 × 30 min and mounted on charged slides

using an aqueous mounting solution (Immu-Mount; Thermo Scientific #9990402). Primary antibodies were used in following dilutions; rabbit anti-PV (1:2000; Swant #25), rabbit anti-CR (1:2000; Swant #7697), rabbit anti-SST (1:2000; Peninsula #T4103), mouse anti-Rln (1:500; Millipore #MAB5364), rabbit anti-NPY (1:8000; Immunostar #22940), rabbit anti-VIP (1:8000; Immunostar #20077).

Interneuron cell counts

Neurons were imaged for Cy5, Tom, GFP, and DAPI fluorescence using a Zeiss LSM710 confocal laser-scanning microscope. Cells were counted from 3 sections of each hemisphere spanning the rostral, medial, and caudal zone of the hippocampus using ImageJ software (Rasband, National Institutes of Health, USA). The quantification areas in somatosensory cortex and hippocampus were selected as follows: 1.0 mm² of cortex through layers I–VI starting approximately 1.0 mm distance from the brain midline and 0.5 mm² of CA1. The numbers of cells counted in *TM^{1z}*.Cre and WT.Cre animals were converted to a percentage based on the average number of total Alexa 647/Cy5⁺ subtype-specific cells that were counted in the WT.Cre brains (which was set at 100%) and data was statistically analyzed. Since there was variation in neuron counts between left and right hemispheres in the adult brains, each hemisphere was counted independently. To determine changes in interneuron morphology, we analyzed interneuron cell size/area containing puncta. To this end, z-stack images of triple-labeled cells were obtained from the somatosensory cortex and hippocampal CA1 region, and the numbers of dense puncta in a 50 µm diameter around the soma (all dense particles >1 µm) was counted from the Alexa 647/Cy5⁺ subtype-specific channel (purple channel).

Statistical analysis

Statistical analysis was performed using two-tailed unpaired student's t-test. All graphs were made in GraphPad Prism 7 software. The number of mouse brains are indicated in each figure legend, and data in all graphs are represented as mean ± SEM (standard error of the mean). A P-value <0.05 was considered as a significant difference between means. A range of P-values in each comparison are simplified by asterisks in each graph (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001), and the exact P-value is reported in the text.

Results

We assessed the population of inhibitory neurons in *Dlx1/2*.Cre containing animals in WT and *TM^{1z}* conditional mice in which *ephrin-B* (*Efnb1/b2/b3*) are deleted. We used several antibody markers for distinct interneuron subtypes including PV, SST, CR, Rln, NPY, and VIP. Cell populations were analyzed for Alexa 647/Cy5⁺ immunofluorescence to identify the distinct interneuron subtype being assessed, Tom⁺ fluorescence to assess whether a cell was exposed to *Dlx1/2*.Cre activity, and GFP⁺ fluorescence to assess whether a cell was of GAD67-GFP or GAD65-GFP group. We divided somatosensory cortex as layers I/II (molecular and external granular layers), III/IV (external pyramidal and dense internal granular layers), and V/VI (internal pyramidal and multiform layers), and hippocampal CA1 layers as stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and stratum lacunosum (SL) sublayers (Figure 1a). These areas are where we previously documented

significant reductions in inhibitory interneurons from $TM^{\Delta Z}$.Cre mice compared to Cre-negative $TM^{\Delta Z}$ mice and WT.Cre animals (Talebian et al., 2017).

To determine interneuron subtype defects in $TM^{\Delta Z}$.Cre mice, we analyzed populations based on combinations of the three independent Cy5, Tom, and GFP markers: (1) The total number of Cy5⁺ subtype-positive cells (e.g PV⁺, purple columns). This group can be Cre-exposed or Cre-unexposed fractions regardless of the GAD-GFP. (2) The portion of subtype-positive cells that were also exposed to the Dlx1/2.Cre driver regardless of the GAD-GFP (e.g PV⁺/Tom⁺, red columns). (3) The portion of subtype-positive cells that co-expressed a GAD-GFP regardless of whether they were exposed to Cre or not (e.g PV⁺/GFP⁺, green columns). (4) The portion of subtype-positive cells that were both exposed to Cre and showed GAD-GFP expression (e.g PV⁺/Tom⁺/GFP⁺, yellow columns). (5) Finally, the portion of subtype-positive cells that neither were exposed to Cre nor expressed GAD-GFP were identified (e.g PV⁺/Tom⁻/GFP⁻, gray columns). The average number of all cell counts (the absolute numbers) are shown in Supplementary Table 1 and scatter plots of the data that go with Figures 1–6 are shown in Supplementary Figures 1–6.

PV subtype is reduced in the cortex and hippocampal CA1 of $TM^{\Delta Z}$.Cre mice

PV is a calcium binding protein acting as a calcium sensor and marks the most abundant subtype of cortical interneurons. PV⁺ interneurons are derived from MGE progenitor cells and co-express GAD67. They represent basket/chandelier cell morphology and are most abundant in cortical layer V/VI. Representative confocal images of our PV analysis is shown in Figure 1b and cell number quantification in Figure 1c and Supplementary Figure 1. Based on total PV⁺ cell numbers in WT.Cre (purple columns), this subtype was distributed across the cortex and hippocampal CA1 region with a higher level in cortical layers V/VI (the 2nd higher is layers III/IV) and in hippocampal SP layer (the 2nd higher is SO layer).

Quantification of cell counts indicated the majority of PV⁺ cells in the somatosensory cortex and hippocampal CA1 were exposed to Dlx1/2.Cre in the WT.Cre and $TM^{\Delta Z}$.Cre mice (Figure 1, red columns, PV⁺/Tom⁺), expressed GAD67-GFP (green columns, PV⁺/GAD67⁺), and were both exposed to Cre and expressed GAD67-GFP (yellow columns, PV⁺/Tom⁺/GAD67⁺), leaving a low level of PV⁺ cells that were not exposed to Cre or expressed GAD67-GFP (gray columns, PV⁺/Tom⁻/GAD67⁻). The numbers of PV⁺/Tom⁺/GAD67⁺ triple-labeled cells were significantly reduced in all cortical layers and in SO, SP & SL layers of hippocampal CA1 in $TM^{\Delta Z}$.Cre brains compared to WT.Cre (6.55%±0.88 vs 11.07%±0.78, P value=0.0014 in LI/II; 14.17%±2.09 vs 19.85%±1.05, P value=0.0153 in LIII/IV; 19.67%±2.22 vs 27.21%±1.62, P value=0.0115 in LV/VI; 40.39%±5.02 vs 58.13%±3.1, P value=0.0051 in total cortex; 10.62%±3.03 vs 22.51%±2.53, P value=0.0077 in SO; 18.29%±4.93 vs 30.27%±3.2, P value=0.0468 in SP; 1.78%±0.7 vs 5.14%±1, P value=0.0242 in SL; and 33.73%±4.81 vs 63.54%±3.87, P value=0.0001 in total CA1). Accordingly, the total number of PV⁺ cells (purple columns) were significantly reduced in cortical layers I/II (15.26%±0.82 vs 18.96%±1.1, P value=0.0253) and total hippocampal CA1 (72.64%±3.69 vs 100%±5.44, P value=0.0016). No change in numbers of PV⁺/Tom⁻/GAD67⁻ interneurons were detected between the $TM^{\Delta Z}$.Cre and WT.Cre mice.

SST subtype is changed mildly in cortex but remarkably in hippocampal CA1 of *TM^z.Cre* mice

SST expressing interneurons are the second most abundant cortical inhibitory neuron. They are derived from MGE progenitor cells and co-express GAD67. Most SST interneurons show a Martinotti cell morphology and are most abundant in layer V of the cortex and SO layer of hippocampal CA1. Representative confocal images of our SST analysis and cell number quantification is shown in Figure 2 and Supplementary Figure 2. Based on total SST⁺ cells in WT.Cre (purple columns), this subtype appeared distributed across cortex and hippocampal CA1 region with a higher level in cortical layers V/VI (the 2nd higher is layers III/IV) and in hippocampal SO layer (the 2nd higher is SP layer).

Quantification of SST⁺ interneurons indicated that the majority of these cells were exposed to Dlx1/2.Cre in the WT.Cre and *TM^z.Cre* mice (Figure 2, red columns, SST⁺/Tom⁺). However, unlike the PV subtype, only ~25% of total SST⁺ cells expressed GAD67-GFP (green columns, SST⁺/GAD67⁺), and even fewer of them were exposed to Cre (yellow columns, SST⁺/Tom⁺/GAD67⁺). This data shows a high number of other undetermined, SST⁺/Tom⁺/GAD67⁻ cells. Nevertheless, the number of SST⁺/Tom⁺/GAD67⁺ triple-labeled cells (yellow columns) were reduced mildly but significantly in cortical layer I/II and to a greater extent in hippocampal SO layer in the *TM^z.Cre* mice compared to the WT.Cre controls (4.11%±0.88 vs 6.15%±0.32, P value=0.0305 in cortical layer I/II; 16.37%±2.47 vs 35.02%±5.07, P value=0.0077 in SO layer; 21.38%±2.67 vs 44.44%±5.83, P value=0.0044 in total CA1). Surprisingly, the total number of SST⁺ cells (purple columns) either remained unchanged in several layers of cortex and hippocampal CA1 or even increased significantly, as observed in the SO and SR regions of CA1 in *TM^z.Cre* compared to WT.Cre (114.69%±8.54 vs 81.48%±7.61, P value=0.0103 in SO; 10.44%±1.92 vs 4.38%±1.51, P value=0.0227 in SR; 140.91%±10.27 vs 100%±8.64, P value=0.0073 in total CA1). This higher level of total SST⁺ cells appeared to be due to significant higher numbers of SST⁺/Tom⁻/GAD67⁻ cells (gray columns) in hippocampal SO layer (39.65%±5.38 vs 18.18%±2.76, P value=0.0017) and total CA1 (46.46%±5.51 vs 21.55%±3.06, P value=0.0007). Although these Cre-unexposed GAD67-negative cells are also increased significantly in cortical layers III/IV and V/VI (6.85%±0.94 vs 4.11%±0.78, P value=0.0379 in layer III/IV; and 12.82%±2.34 vs 6.61%±1.41, P value=0.0304 in layer V/VI), the total numbers of SST⁺ cells remained unchanged in these layers.

CR subtype is reduced in both GAD67 and GAD65 groups in *TM^z.Cre* mice

CR expressing interneurons are derived from both MGE and CGE progenitors, and can express either GAD67 or GAD65. CR interneurons present mostly bipolar or double bouquet cell morphology distributed across cortical layers II–VI. Representative confocal images of our CR analysis and cell number quantification is shown in Figure 3 and Supplementary Figure 3. Based on total CR⁺ cells in WT.Cre (purple columns), this subtype was distributed across cortex and hippocampal CA1 with a higher level in cortical layers I/II (the 2nd higher is layers III/IV) and in hippocampal SP layer (the 2nd higher is SR layer).

Quantification indicated the majority of CR⁺ cells in WT.Cre and *TM^z.Cre* mice were exposed to Dlx1/2.Cre (Figure 3, red columns, CR⁺/Tom⁺), labeled with GAD67-GFP or

GAD65-GFP (green columns), and were both exposed to Cre and expressed a GAD-GFP (yellow columns). The number of CR⁺/Tom⁺/GAD67⁺ triple-labeled cells were significantly reduced in cortical layers I/II & III/IV and also in hippocampal SO & SR layers of *TM^z.Cre* mice compared to WT.Cre mice (16.36%±2 vs 24.15%±2.26, P value=0.0265 in LI/II; 11.3%±1.4 vs 16.1%±1.06, P value=0.0124 in LIII/IV; 27.77%±3.8 vs 47.89%±3.67, P value=0.0017 in total cortex; 5.82%±1.09 vs 11.72%±1.9, P value=0.0302 in SO; 4.67%±1.26 vs 11.3%±2.21, P value=0.0357 in SR; and 30.76%±3.01 vs 48.82%±4.23, P value=0.0058 in total CA1). The number of CR⁺/Tom⁺/GAD65⁺ triple-labeled cells were also reduced mainly in cortical layers I/II & III/IV (4.31%±1.57 vs 10.08%±1.84; P value=0.0342 in LI/II; 2.69%±0.92 vs 6.06%±1.21, P value=0.0497 in LIII/IV; and 8.38%±2.92 vs 19.07%±3.7, P value=0.0446 in total cortex) and in SR layer of hippocampus (0%±0 vs 4.09%±1.16, P value=0.0063 in SR and 6.23%±2.13 vs 18.42%±3.8, P value=0.0189 in total CA1). Accordingly, the total number of CR⁺ cells (purple columns) were reduced in some layers of cortex and hippocampus, significantly in cortex layer I/II (39.71%±2.4 vs 50.93%±2.68, P value=0.0051) and hippocampal SR layer (16.64%±1.96 vs 25%±2.64, P value=0.0235), with a significant reduction in total cortex (83.3%±7.67 vs 100%±4.06, P value=0.0458) and total CA1 (86.42%±5.08 vs 100%±4.33, P value=0.0491). Similar to the SST analysis, the reduced numbers of triple-labeled CR interneurons observed *TM^z.Cre* mice was partially offset/counterbalanced by significant increases in numbers of CR⁺/Tom⁻/GAD⁻ cells (gray columns) in both the cortex and hippocampus.

NPY subtype cortical layering and hippocampal CA1 population is disrupted in *TM^z.Cre* mice

Interneurons positive for NPY exhibit similar features as CR subtypes; they are derived from both MGE and CGE progenitors, can express either GAD67 (major fraction) or GAD65 (minor fraction), and present mostly bipolar or double bouquet cell morphology. Representative confocal images of our NPY analysis and cell number quantification is shown in Figure 4 and Supplementary Figure 4. Based on total NPY⁺ cells in WT.Cre (purple columns), this subtype was distributed across cortex and hippocampal CA1 with a higher level in cortical layers I/II (the 2nd higher is layers V/VI) and in hippocampal SO layer (the 2nd higher is SR layer).

We found that the majority of NPY⁺ cells in WT.Cre and *TM^z.Cre* mice were also exposed to Dlx1/2.Cre (Figure 4, red columns), labeled with GAD67-GFP or GAD65-GFP (green columns), and were both exposed to Cre and expressed a GAD-GFP (yellow columns). The quantification of NPY⁺/Tom⁺/GAD⁺ triple-labeled cells (yellow columns) revealed the most significant changes were in the hippocampal CA1 of *TM^z.Cre* mice compared to WT.Cre exclusively affecting the GAD67 group. Here, the number of NPY⁺/Tom⁺/GAD67⁺ cells were significantly reduced in hippocampal CA1 layers SO and SR of *TM^z.Cre* mice compared to WT.Cre mice (11.26%±2.32 vs 34.34%±2.74, P value<0.0001 in SO; and 10.32%±1.98 vs 17.73%±2.01, P value=0.0284 in SR). In the cortex, the distribution of these triple-labeled cells in *TM^z.Cre* mice was altered such that they were significantly decreased in layers I/II (19.05%±3.16 vs 33.19%±4.09, P value=0.0302) and increased in layers III/IV (24.29%±3.89 vs 15.4%±0.85, P value=0.0134). Total NPY⁺ cells (purple columns) were also significantly increased in layers III/IV and V/VI in *TM^z.Cre* mice

(42.51%±3.63 vs 21.09%±0.78, P value<0.0001 in LIII/IV; 45.16%±1.78 vs 35.42%±1.93, P value=0.0011 in LV/VI; and 135.38%±6.62 vs 100%±5.65, P value=0.0003 in total cortex), which was associated with an increase in NPY⁺/GAD67⁺ cells (green columns; 43.19%±5 vs 19.68%±1.08, P value<0.0001 in LIII/IV; and 119.29%±10.11 vs 91.83%±5.33, P value=0.0189 in total cortex) and undetermined, NPY⁺/Tom⁻/GAD⁻ cells (gray columns; 13.59%±4.3 vs 1.82%±0.33, P value=0.0041 in LI/II; 9.24%±2.11 vs 1.55%±0.29, P value=0.0003 in LIII/IV; 12.19%±2.65 vs 2.29%±0.48, P value=0.0003 in LV/VI; and 35%±8.85 vs 5.66%±0.71, P value=0.0007 in total cortex).

RIn subtype is mildly reduced in cortex of GAD67 group but remarkably reduced in hippocampal CA1 of both GAD67/65 groups in *TM^{fl}*.Cre mice

RIn-positive interneurons exhibit similar features as CR and NPY subtypes. They are also derived from both MGE and CGE progenitors, express either GAD67 (major fraction) or GAD65 (minor fraction), and most present a bipolar or double bouquet cell morphology. Representative confocal images of our RIn analysis and cell number quantification is shown in Figure 5 and Supplementary Figure 5. Based on total RIn⁺ cells in WT.Cre (purple columns), the RIn-positive subtype was distributed across cortex and hippocampal CA1 with a higher level in cortical layers I/II (the 2nd higher is layers III/IV), and in hippocampal SL layer (the 2nd higher is SO layer).

We found that the majority of total RIn⁺ cells in WT.Cre and *TM^{fl}*.Cre mice were also exposed to Cre (Figure 5, red columns), labeled with GAD67-GFP or GAD65-GFP (green columns), and were both exposed to Cre and expressed a GAD-GFP (yellow columns), leaving a low level of RIn⁺/Tom⁻/GAD⁻ cells in the WT.Cre brains (gray columns). In *TM^{fl}*.Cre mice, the number of RIn⁺/Tom⁺ cells (red columns) were reduced significantly in all layers of cortex (21.33%±3.15 vs 32.72%±2.35, P value=0.0065 in LI/II; 18.49%±2.39 vs 27.28%±2.13, P value=0.0112 in LIII/IV; 16.03%±2.91 vs 26.5%±2.23, P value=0.0074 in layers V/VI; and 55.86%±8.14 vs 86.5%±6.2, P value=0.0052 in total cortex). Consistent with this, the number of RIn⁺/Tom⁺/GAD67⁺ triple-labeled cells was significantly reduced in cortex layer I/II in *TM^{fl}*.Cre mice compared to WT.Cre (22.16%±4.12 vs 32.85%±1.05, P value=0.0134), while RIn⁺/Tom⁺/GAD65⁺ cells showed a trend towards reduced numbers. The reduced numbers of RIn⁺/Tom⁺ and RIn⁺/Tom⁺/GAD⁺ cells observed in the *TM^{fl}*.Cre mice were counterbalanced by a corresponding increase in numbers of RIn⁺/Tom⁻/GAD⁻ cells (gray columns). In the hippocampus, the numbers of RIn⁺/Tom⁺/GAD67⁺ triple-labeled cells were significantly reduced in SO & SL layers of CA1 region in *TM^{fl}*.Cre mice (4.89%±0.7 vs 17.41%±2.11, P value=0.0001 in SO; 20%±2.06 vs 30.69%±3.13, P value=0.0160 in SL; and 28.10%±2.68 vs 53.78%±3.88, P value=0.0001 in total CA1), whereas the numbers of RIn⁺/Tom⁺/GAD65⁺ triple-labeled cells were significantly decreased in SL layer (5.65%±1.85 vs 11.87%±1.26, P value=0.0200) with an overall significant decrease in total CA1 (6.01%±1.59 vs 18.28%±2.97, P value=0.0140).

VIP subtype is little changed in *TM^{fl}*.Cre mice

Interneurons positive for VIP are derived from CGE progenitor cells. They express GAD65 and present mostly bipolar or double bouquet cell morphology. Representative confocal images of our VIP analysis and cell number quantification is shown in Figure 6 and

Supplementary Figure 6. Similar to CR, based on total VIP⁺ cells in WT.Cre (purple columns), this subtype appeared distributed across the cortex and hippocampal CA1 with a higher level in cortical layers I/II (the 2nd higher is layers III/IV) and in hippocampal SP layer (the 2nd higher is SR layer).

Quantification of the data indicate that majority of VIP⁺ cells in the cortex and hippocampus were exposed to Cre (Figure 6, red columns). However, only about 20–30% of the total VIP⁺ cells expressed GAD65-GFP (green columns), most of which were also exposed to Cre (yellow columns). This suggests that a high number of other undetermined, Cre-exposed, GAD65-negative interneurons in the VIP subtype, and is similar to what was observed in the SST analysis with GAD67-GFP reporter. Nevertheless, while the numbers of VIP⁺/Tom⁺/GAD65⁺ triple-labeled cells (yellow columns) and VIP⁺/Tom⁺/GAD65⁻ cells (red columns) were unchanged throughout the cortex in *TM^z.Cre* mice relative to WT.Cre, they did show a trend to reduction in all layers. These reductions, however, were counterbalanced by significant increases in the number of VIP⁺/Tom⁻/GAD65⁻ cells (gray columns) in the cortex of *TM^z.Cre* mice (18.12%±3.5 vs 1.97%±0.51, P value=0.0020 in layer I/II; 9.81%±2.76 vs 1.97%±0.53, P value=0.0330 in layer III/IV; 6.35%±1.18 vs 11.57%±0.44, P value=0.0057 in layer V/VI; and 34.27%±6.32 vs 5.51%±1.02, P value=0.0022 in total cortex). Analysis of VIP⁺ interneurons in the CA1 region did not reveal any significant changes.

Reduced interneuron soma size/area in *TM^z.Cre* mice

We used the punctate staining observed with subtype-specific antibodies to assess for abnormalities in interneuron morphology in *TM^z.Cre* brains compared to WT.Cre controls. This assessment indirectly measures the area occupied by a cell soma and its proximal cell processes containing dendrites and the primary axon (Brenneman and Maness, 2008). We counted the number of distinct subtype-specific puncta > 1 µm in size around cell soma of triple-labeled cells using the Cy5⁺ channel. To best ensure that puncta originated from an individual triple-labeled cell soma, we counted the number of puncta in a limited 50 µm diameter around that individual cell soma to restrict analysis to a minimal distance surrounding that cell. We also avoided quantifying from areas that had other nearby triple-labeled cells present in order to limit contribution from adjacent subtype labeled cells. We performed punctate analysis for PV and CR subtypes, and the average number of perisomatic particle (puncta) counts per cell are shown in Supplementary Table 2.

The punctate PV⁺ staining of PV⁺/Tom⁺/GAD67⁺ triple-labeled cells (Figure 7) revealed a significant reduction in cell size/area in both the cortex and hippocampal CA1 of these interneurons in *TM^z.Cre* brains compared to WT.Cre (76.06%±6.55 vs 100%±5.72, P value=0.0087 in cortex and 82.05%±4.7 vs 100%±5.2, P value=0.0191 in hippocampal CA1). The puncta staining of CR⁺/Tom⁺/GAD⁺ interneurons (Figure 8) was also reduced in *TM^z.Cre* compared to WT.Cre mice in both GAD67 group (69.29%±4.36 vs 100%±6.27, P value=0.0006 in cortex and 65%±12.24 vs 100%±5.04, P value=0.0049 in hippocampal CA1) and GAD65 group (37%±6.51 vs 100%±9.08, P value<0.0001 in cortex and 52.96%±9.03 vs 100%±16.55, P value=0.0294 in hippocampal CA1).

Discussion

In this study, we analyzed inhibitory interneurons using specific antibody probes to detect PV, CR, SST, NPY, Rln, and VIP subtypes to determine if loss of ephrin-B expression affects distinct interneuron subpopulations and to obtain a better understanding of the roles for ephrin-B within GABAergic neurons. For this purpose, we used *Dlx1/2.Cre* driver with Tom indicator to identify *ephrin-B* deleted interneurons, and also utilized GAD65-GFP and GAD67-GFP reporters to brightly label the two major classes of GABAergic interneurons. GAD67-GFP brightly labels several interneuron subtypes including PV, SST, CR, NPY, and Rln, while GAD65-GFP labels other subtypes of CR, NPY, Rln, as well as VIP (Fukuda et al., 1997; Lopez-Bendito et al., 2004; Tamamaki et al., 2003; Wierenga et al., 2010). We previously determined that all three ephrin-Bs, especially ephrin-B2, are expressed in ~90% of inhibitory interneurons of the GAD67-GFP and GAD65-GFP cells in the embryonic brain and that ephrin-B expression in interneurons is maintained in the adult (Talebian et al., 2017). Here, we now were able to reliably identify and score interneurons using the two distinct GAD67-GFP and GAD65-GFP reporters, the Tom indicator of Cre activity, and specific antibodies that recognize distinct interneuron subtypes. We find that many of these subtypes are affected by *Dlx1/2.Cre* mediated deletion of ephrin-B, resulting in reduced numbers and/or layering abnormalities of interneurons in the somatosensory cortex and hippocampal CA1. Further, analysis of cell size/area for PV and CR show a reduced complexity compared to the WT.Cre control brains, indicating potential roles in elaboration of proximal cell processes, dendrites and axons, following ephrin-B deletion.

Disruption in various GABAergic interneuron subtypes occurs in a variety of neuropsychiatric disorders such as schizophrenia (reported for PV, SST, CR, Rln and NPY subtypes), autism spectrum disorders (reported for PV, Rln, NPY subtypes), epilepsy or seizure-associated disorders (reported for PV, SST, Rln, NPY subtypes), Alzheimer's (reported for PV, Rln subtypes), and mood disorders (reported for PV subtype) (Brisch et al., 2015; Colmers and El Bahh, 2003; Faux et al., 2012; Folsom and Fatemi, 2013; Lewis, 2009; Marin, 2012; Toth and Magloczky, 2014; Urban-Ciecko and Barth, 2016; Yu et al., 2016). It has been shown that PV⁺ subtype is also disrupted in various ASD mouse models (Hensch, 2005) and cognitive dysfunction in Alzheimer's models (Verret et al., 2012). Interestingly, potential roles for ephrin/Eph signaling in some of these disorders have been proposed (Faux et al., 2012; Pasquale, 2008; Rudolph et al., 2014; Steinecke et al., 2014; Villar-Cervino et al., 2015; Wen et al., 2010; Wurzman et al., 2015; Zimmer et al., 2011). Consistent with this notion, we found that ephrin-B deleted interneurons from diverse subtypes of PV, SST, CR, Rln and NPY were disrupted in either cortex and/or various layers of hippocampal CA1 in the *TM^{fl}.Cre* mice which was previously shown to have high cortical excitability and seizure activity (Talebian et al., 2017).

We investigated here the abundance of interneuron subtypes in *Dlx1/2.Cre* containing WT and *TM^{fl}* mice by analyzing GAD67/GAD65 markers in combination with Cre exposure (yellow columns) to determine subtypes based on their birth origin in part from embryonic MGE or CGE areas and by analysis focused on Cre exposure regardless of GAD expression (red columns). Our results indicate that the subtypes PV (GAD67), CR (GAD67/GAD65) and Rln (GAD67) are affected in the cortex of *TM^{fl}.Cre* mice, while PV

(GAD67), SST (GAD67), CR (GAD67/GAD65), NPY (GAD67), and Rln (GAD67/GAD65) subtypes are affected in the hippocampal CA1 region (Table 1). Our studies allowed for some additional interesting observations. First, the analysis of PV subtype was most straightforward. The $TM^{\Delta Z}$.Cre brains showed clear and significant reductions in total PV⁺ cells (purple columns), in PV⁺/Tom⁺ Cre-exposed cells (red columns), and in PV⁺/Tom⁺/GAD67⁺ Cre-exposed, GAD67-expressing cells (yellow columns). Plus, there were no increases in PV⁺/Tom⁻/GAD67⁻ Cre-unexposed, GAD67-negative cells (gray columns) to counterbalance the ephrin-B deleted interneurons. Second, in both WT.Cre and $TM^{\Delta Z}$.Cre brains the majority of SST⁺ cells were not GAD67⁺, especially in the cortex, and the majority of VIP⁺ cells were not GAD65⁺. However, even though negative for their corresponding GAD-GFP, most of the SST-positive and VIP-positive cells were exposed to Dlx1/2.Cre and thus identified as bona fide interneurons. Third, excluding the PV subtype, we observed corresponding increases in numbers of Cre-unexposed, GAD-negative cells only in the $TM^{\Delta Z}$.Cre brains that appeared to counterbalance/offset the reductions in Cre-exposed cells (Table 1). In the cortex this was observed in all SST, CR, NPY, Rln, and VIP subtypes, whereas in the hippocampal CA1 region only SST, CR, and NPY subtypes were strongly affected. We hypothesize that there must be some sort of compensating homeostatic mechanism at play for these interneuron subtypes that allows for the expansion of the undeleted ephrin-B-positive pool of interneurons in the $TM^{\Delta Z}$.Cre brains that escaped Dlx1/2.Cre mediated recombination.

We previously reported the loss of presynaptic/postsynaptic ephrin-Bs in excitatory axon pruning, dendritic branching, and synapse formation in the hippocampus (Xu and Henkemeyer, 2009, Xu et al., 2011; Xu and Henkemeyer, 2012). Here, we additionally analyzed the complexity of inhibitory GABAergic neurons for PV and CR subtypes to study cellular morphologies of Cre-exposed ephrin-B deleted interneurons in the $TM^{\Delta Z}$.Cre mice. In triple-labeled cells of Cre-exposed with either GAD67-GFP or GAD65-GFP and each subtype individually, we observed that PV and CR interneuron subtypes in cortex and hippocampus display significantly reduced axonal/dendritic complexity in the $TM^{\Delta Z}$.Cre mice.

It remains to further investigate how ephrin-B/EphB interaction is involved in distinct interneuron subtypes. One way to address this question would be to use Cre expressing mice that target distinct subtypes for ephrin-B deletion rather than using the pan inhibitory Dlx1/2.Cre driver. Further investigation is also required to determine if loss of ephrin-B affects interneuron connectivity and synapse formation/function and integration with excitatory neurons to regulate excitatory-inhibitory balance in the cortex and hippocampus. Studies for learning/memory deficits and/or behavioral abnormalities in Dlx1/2.Cre containing $TM^{\Delta Z}$ mice would further address the functional consequences of inhibitory neuron loss of ephrin-B expression. Collectively, our data indicate that the reduction of interneurons in the cortex and hippocampus observed in our previous study (Talebian et al., 2017) is associated with a reduction in several interneuron subtypes and a reduction in the soma complexity. This disruption in a broad range of interneuron subtypes might be responsible for the increased cortical excitability and lethal seizures observed in $TM^{\Delta Z}$.Cre mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TM	triple mutant
GFP	green fluorescent protein
GAD	glutamic acid decarboxylase
GEs	ganglionic eminences
MGE	medial ganglionic eminence
CGE	caudal ganglionic eminence
PV	parvalbumin
SST	somatostatin
CR	calretinin
Rln	reelin
NPY	neuropeptide Y
VIP	vasoactive intestinal polypeptide
WT	wild-type

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Figure 1. Parvalbumin (PV) subtype analysis of WT.Cre and TM^{Lz} .Cre mice

a) Coronal section of adult mouse brain that highlights the area of somatosensory cortex (layers I/II, III/IV, and V/VI) and hippocampal CA1 region (layers SO, SP, SR, SL) that were analyzed in this study.

b) Representative confocal images of brain sections from WT.Cre and TM^{Lz} .Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD67-GFP (GFP, green fluorescence), and anti-PV antibody signal (purple fluorescence). scale bar = 100 μ m.

c) Quantification of PV⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for brain hemispheres: WT.Cre (12), TM^{Lz} .Cre (8).

Figure 2. Somatostatin (SST) subtype analysis of WT.Cre and *TM^{Lz}*.Cre mice
a) Representative confocal images of brain sections from WT.Cre and *TM^{Lz}*.Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD67-GFP (GFP, green fluorescence), and anti-SST antibody signal (purple fluorescence). scale bar = 100 μ m.
b) Quantification of SST⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for brain hemispheres: WT.Cre (10), *TM^{Lz}*.Cre (8).

Figure 3. Calretinin (CR) subtype analysis of WT.Cre and TM^{1z} .Cre mice
a) Representative confocal images of brain sections from WT.Cre and TM^{1z} .Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD67-GFP (GFP, green fluorescence) or GAD65-GFP (GFP, green fluorescence), and anti-CR antibody signal (purple fluorescence). scale bar = 100 μ m.
b) Quantification of CR⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for GAD67-GFP brain hemispheres: WT.Cre (12), TM^{1z} .Cre (8); for GAD65-GFP brain hemispheres: WT.Cre (10), TM^{1z} .Cre (8).

Figure 4. Neuropeptide Y (NPY) subtype analysis of WT.Cre and *TM1^z*.Cre mice
a) Representative confocal images of brain sections from WT.Cre and *TM1^z*.Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD67-GFP (GFP, green fluorescence) or GAD65-GFP (GFP, green fluorescence), and anti-NPY antibody signal (purple fluorescence). scale bar = 100 μ m.
b) Quantification of NPY⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for GAD67-GFP brain hemispheres: WT.Cre (10), *TM1^z*.Cre (6); for GAD65-GFP brain hemispheres: WT.Cre (8), *TM1^z*.Cre (8).

Figure 5. Reelin (Rln) subtype analysis of WT.Cre and *TM1z*.Cre mice

a) Representative confocal images of brain sections from WT.Cre and *TM1z*.Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD67-GFP (GFP, green fluorescence) or GAD65-GFP (GFP, green fluorescence), and anti-Rln antibody signal (purple fluorescence). scale bar = 100 μ m.

b) Quantification of Rln⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for GAD67-GFP brain hemispheres: WT.Cre (10), *TM1z*.Cre (8); for GAD65-GFP brain hemispheres: WT.Cre (6), *TM1z*.Cre (4).

Figure 6. Vasoactive intestinal polypeptide (VIP) subtype analysis of WT.Cre and TM^{lz} .Cre mice
a) Representative confocal images of brain sections from WT.Cre and TM^{lz} .Cre mice for Cre-exposed cells (Tom, red fluorescence), GAD65-GFP (GFP, green fluorescence), and anti-VIP antibody signal (purple fluorescence). scale bar = 100 μ m.
b) Quantification of VIP⁺ cells in cortex (top) and hippocampal CA1 (bottom). N values for brain hemispheres: WT.Cre (6), TM^{lz} .Cre (8).

Figure 7. Perisomatic puncta assessment in PV subtype of WT.Cre and *TM1^z*.Cre mice
a) Representative confocal images of PV⁺/Tom⁺/GAD67⁺ triple-labeled cells in WT.Cre and *TM1^z*.Cre brains. The Tom and GFP channels confirm triple-labeling and the purple Alexa 647/Cy5⁺ channel was used to count punctate particles > 1 μm. scale circle = 50 μm diameter.
b) Quantification of PV⁺ puncta counts from WT.Cre and *TM1^z*.Cre brains. N values: WT.Cre (12 brains/36 cells), *TM1^z*.Cre (8 brains/24 cells).

Figure 8. Perisomatic puncta assessment in CR subtype of WT.Cre and TM^{lz} .Cre mice

a) Representative confocal images of $CR^+/Tom^+/GAD^+$ triple-labeled cells in both GAD67-GFP and GAD65-GFP groups in WT.Cre and TM^{lz} .Cre brains. The Tom and GFP channels confirm triple-labeling and the purple Alexa 647/Cy5⁺ channel was used to count punctate particles > 1 μm . scale circle = 50 μm diameter.

b) Quantification of CR^+ puncta counts in GAD67 and GAD65 groups from WT.Cre and TM^{lz} .Cre brains. N values for GAD67-GFP: WT.Cre (12 brains/35 cells), TM^{lz} .Cre (8 brains/24 cells); for GAD65-GFP: WT.Cre (10 brains/30 cells), TM^{lz} .Cre (8 brains/24 cells).

Table 1

Summary of interneuron subtype abnormalities observed in *TM^{hZ}.Cre* brains.

Subtype	GAD-GFP	Cortex reduced ^a	Cortex counterbalanced ^b	CA1 reduced ^a	CA1 counterbalanced ^b
PV	67	+++++	no	+++++	no
	67	++	++	+++	+++
CR	67	+++++	+++	+++++	+++
	65	+++++		+++++	
NPY	67	++ (abnormal layers)	+++	++++	+++
	65	no		no	
Rln	67	++	+++	+++++	no
	65	+		+++++	
VIP	65	+	+++	+	+

^aThe strength of the phenotype observed for each interneuron subtype that exhibited a reduced cell population in the *TM^{hZ}.Cre* brains was assessed by the following criteria:

+++++ Significantly reduced cell numbers that affected red, yellow, and either purple or green groupings in both the total area assessed and in specific layers.

+++ Significantly reduced cell numbers that affected both red and yellow groupings in both the total area assessed and in specific layers.

++ Significantly reduced cell numbers that affected yellow grouping in both the total area assessed and in specific layers.

+ Significantly reduced cell numbers that affected yellow grouping in either the total area assessed or in specific layers.

no A non-significant trend towards reduced cell numbers that affected yellow grouping in both the total area assessed and in specific layers.

^bThe strength of the phenotype observed for each interneuron subtype that exhibited a counterbalancing increase in the gray cell grouping numbers in the *TM^{hZ}.Cre* brains was assessed by the following criteria:

++++ Significantly increased cell numbers in both the total area assessed and in specific layers.

+++ Significantly increased cell numbers in either the total area assessed or in specific layers.

+ A non-significant trend towards increased cell numbers in either the total area assessed or in specific layers.