Cellular/Molecular

# Regulation of Spine Formation by ErbB4 in PV-Positive Interneurons

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**The trophic factor neuregulin 1 (*Nrg1*) and its receptor *ErbB4* are schizophrenia candidate genes. NRG1-ErbB4 signaling was thought to regulate spine formation and function in a cell-autonomous manner. Yet, recent studies indicate that ErbB4 expression is largely restricted to GABAergic interneurons and is very low or absent in pyramidal cells. Here, we generated and characterized cell type-specific *ErbB4* mutant and transgenic mice. Spine density and the number of excitatory synapses were unaltered by neither deletion nor overexpression of ErbB4 in pyramidal neurons. However, spine density and excitatory synapse number were reduced in PV-*ErbB4*$^{-/-}$ mice where ErbB4 was selectively ablated in parvalbumin-positive GABAergic interneurons. Concurrently, basal glutamate transmission was impaired in PV-*ErbB4*$^{-/-}$ mice, but not in mice where ErbB4 was deleted or overexpressed in pyramidal neurons. Our results demonstrate a role of ErbB4 in PV-positive interneurons for spine formation in excitatory neurons.**

*Key words:* **neuregulin; ErbB4; GABAergic; glutamatergic; spine; schizophrenia**

## Introduction

Schizophrenia is a neural developmental disorder characterized by impaired perception, cognition, and motivation (Weinberger, 1987; Lewis and Levitt, 2002). These impairments may result from altered assembly and function of excitatory and inhibitory pathways (Lewis and Sweet, 2009; Yin et al., 2012). Excitatory synapses onto pyramidal neurons are formed by glutamatergic axon terminals onto spines, protrusions from dendritic shafts of pyramidal neurons (Gray, 1959; Harris and Kater, 1994). Consistent with synaptic dysfunction in schizophrenia, recent studies suggest spines as important substrates in pathogenesis of this disorder (Penzes et al., 2011; Glausier and Lewis, 2013).

Neuregulin 1 (NRG1) is a large family of EGF-domain-containing trophic factors that act by activating ErbB receptor tyrosine kinases (Mei and Xiong, 2008). Both *Nrg1* and *ErbB4*, the only ErbB that can function as homodimer, have been identified as schizophrenia susceptibility genes (Stefansson et al., 2002; Yang et al., 2003; Silberberg et al., 2006; Sullivan et al., 2008; Walsh et al., 2008; Shi et al., 2009). Recent studies have revealed that NRG1 and ErbB4 play important roles in neural development as well as synaptic plasticity (Huang et al., 2000; Flames et al., 2004; Woo et al., 2007; Pitcher et al., 2008; Chen et al., 2010; Fazzari et al., 2010; Wen et al., 2010; Ting et al., 2011; Del Pino et al., 2013).

NRG1-ErbB4 signaling has been implicated in spine formation or maturation. Fewer spines were observed in hGFAP::Cre;*ErbB4*f/f mice where ErbB4 was ablated in all neural cells (Barros et al., 2009). However, the underlying mechanisms remain unclear. Treatment with soluble NRG1 increased the number of spines in cultured hippocampal neurons (Barros et al., 2009) but had little effect on the density and size of PSD-95 puncta in cortical pyramidal neurons (Ting et al., 2011). Knockdown of ErbB4 reduced spine density and size in organotypical hippocampal slices (Li et al., 2007). However, the cell-autonomous function of ErbB4 in spine formation deserves a revisit because ErbB4 ablation in pyramidal neurons by viral infection did not alter the density of dendritic spines (Fazzari et al., 2010). Moreover, recent studies indicate that ErbB4 expression is largely restricted to GABAergic interneurons and is very low or absent in pyramidal cells (Vullhorst et al., 2009; Fazzari et al., 2010).

Here we addressed these discrepancies by *in vivo* genetic studies. We found that neither deletion nor overexpression of ErbB4 in pyramidal neurons altered spine density and number of excitatory synapses *in vivo*. However, the spine density and number of excitatory synapses in pyramidal neurons were reduced in PV-*ErbB4*$^{-/-}$ mice where ErbB4 was selectively deleted in PV-positive GABAergic interneurons. These results demonstrate a

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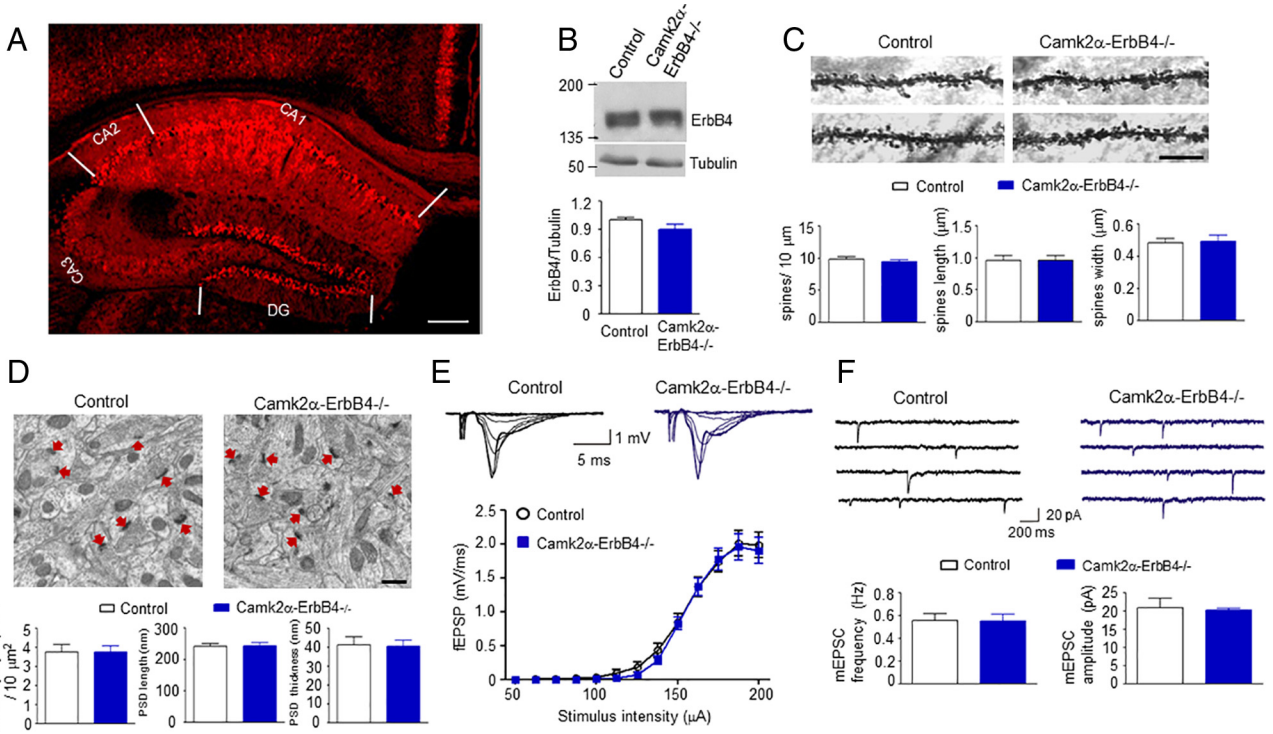


**Figure 1.** Deletion of ErbB4 in pyramidal neurons has no effect on spine formation. ***A***, Cre activity was expressed in forebrain pyramidal neurons in Camk2α::Cre mice. Camk2α::Cre mice were crossed with Rosa-tdTomato mice. Shown were a hippocampal slice of resulting Camk2α::Cre;Rosa-tdTomato mice. Scale bar, 200 μm. ***B***, Similar ErbB4 levels in the hippocampus between Camk2α-*ErbB4*$^{-/-}$ mice and control littermates. Top, Representative blots of ErbB4 and tubulin. Bottom, Quantification data. Expression levels of ErbB4 were normalized by tubulin. ***C***, The density, length, and width of spines were indistinguishable between control and Camk2α-*ErbB4*$^{-/-}$ mice. Top, Representative images. Bottom, Quantitative data. $n = 33$ neurons from 3 mice per group. Scale bar, 10 μm. ***D***, Comparable numbers of excitatory synapses, PSD length, and PSD thickness in the CA1 region between control and Camk2α-*ErbB4*$^{-/-}$ mice. Top, Representative EM images. Bottom, Quantitative data. $n = 108$ images from 3 mice per group. Arrows indicate PSD. Scale bar, 0.5 μm. ***E***, Similar I/O curves of fEPSP at SC–CA1 synapses between control and Camk2α-*ErbB4*$^{-/-}$ mice. $n = 12$ slices from 4 or 5 mice per group. Top, Representative traces of fEPSPs. Bottom, I/O curves. ***F***, No difference in mEPSC frequency and amplitude between control and Camk2α-*ErbB4*$^{-/-}$ mice. Top, Representative traces. Bottom, Quantification data. $n = 9$ cells from 3 mice per group.

role of ErbB4 from PV-positive interneurons in regulating spine formation.

## Materials and Methods

*Generation of mice.* Camk2α-*ErbB4*$^{-/-}$ and PV-*ErbB4*$^{-/-}$ mice were generated as described previously (Chen et al., 2010). Rosa-tdTomato reporter mice were from The Jackson Laboratory (stock number 007909) (Madisen et al., 2010). To create transgenic mice where ErbB4 is overexpressed in pyramidal neurons, full-length human *ErbB4* was cloned into the EcoR V site of the pMM400 plasmid (kindly provided by Dr. Joe Tsien, Georgia Regents University). A NotI fragment containing the transgene was isolated and used for transgenic mouse production. Transgenic C57BL/6-CBA(J) F2 founders, TRE-*ErbB4* mice, were backcrossed with C57BL/6N mice for 6 generations before crossing with Camk2α::tTA mice (The Jackson Laboratory, stock number 007004) to generate bitransgenic Camk2α::tTA;TRE-*ErbB4* mice (cto*ErbB4* for Camk2α promoter driven *t*et-*o*ff *ErbB4*). Mice were housed in rooms with temperature at 23°C in a 12 h light/dark cycle and with food and water available *ad libitum*. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Georgia Regents University. Unless otherwise indicated, 2-month-old male mice were used for all experiments. *ErbB4*f/f mice were used as control for Camk2α-*ErbB4*$^{-/-}$ and PV-*ErbB4*$^{-/-}$ mice. TRE-*ErbB4* mice were used as control for cto*ErbB4* mice.

*Golgi staining.* Golgi staining was performed by using a kit following the manufacturer's protocol (FD NeuroTechnologies). Spines were counted on secondary and tertiary branches of apical dendrites in the stratum radiatum of CA1 hippocampal region. Five 20 μm segments of either secondary or tertiary dendrites were randomly selected. Spine length and width were measured by ImageJ and analyzed by investigators who were blind to genotypes.

*Electron micrograph.* Ultrathin sections of the CA1 region were examined with a JEM 1230 transmission electron microscope (JEOL USA) at 110 kV. Images were collected with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan). Synapses were identified by ultrastructural specializations, including alignment of presynaptic and postsynaptic membranes, presynaptic and postsynaptic thickenings, and clusters of synaptic vesicles. The length and thickness of postsynaptic density (PSD) were analyzed by investigators unaware of genotypes using ImageJ.

*Electrophysiology.* Electrophysiology was performed as described previously (Yin et al., 2013). Briefly, slices were placed in the recording chamber that was perfused (3 ml/min) with ACSF at 32–34°C. Field EPSPs (fEPSP) were evoked in the CA1 stratum radiatum by stimulating Schaffer collaterals (SCs) with a two-concentric bipolar stimulating electrode (FHC) and recorded in current-clamp configuration by the Axon MultiClamp 700B amplifier with ACSF-filled glass pipettes (1–5 MΩ). Test stimuli consisted of monophasic 100 μs pulses of constant currents at a frequency of 0.033 Hz. The strength of synaptic transmission was determined by measuring the initial (10–60% rising phase) slope of fEPSP. Whole-cell recording was aided with infrared optics using an upright microscope equipped with a ×40 water-immersion lens (Olympus, BX51WI) and infrared-sensitive CCD camera. The pipette (input resistance: 4–6 MΩ) solution contained (mM) the following: 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA, 5 QX314, pH 7.35, 285 mOsm. To measure miniature EPSCs (mEPSCs), $GABA_A$ receptor and action potentials were blocked with 20 μM bicuculline methodiode and 1 μM TTX, respectively.

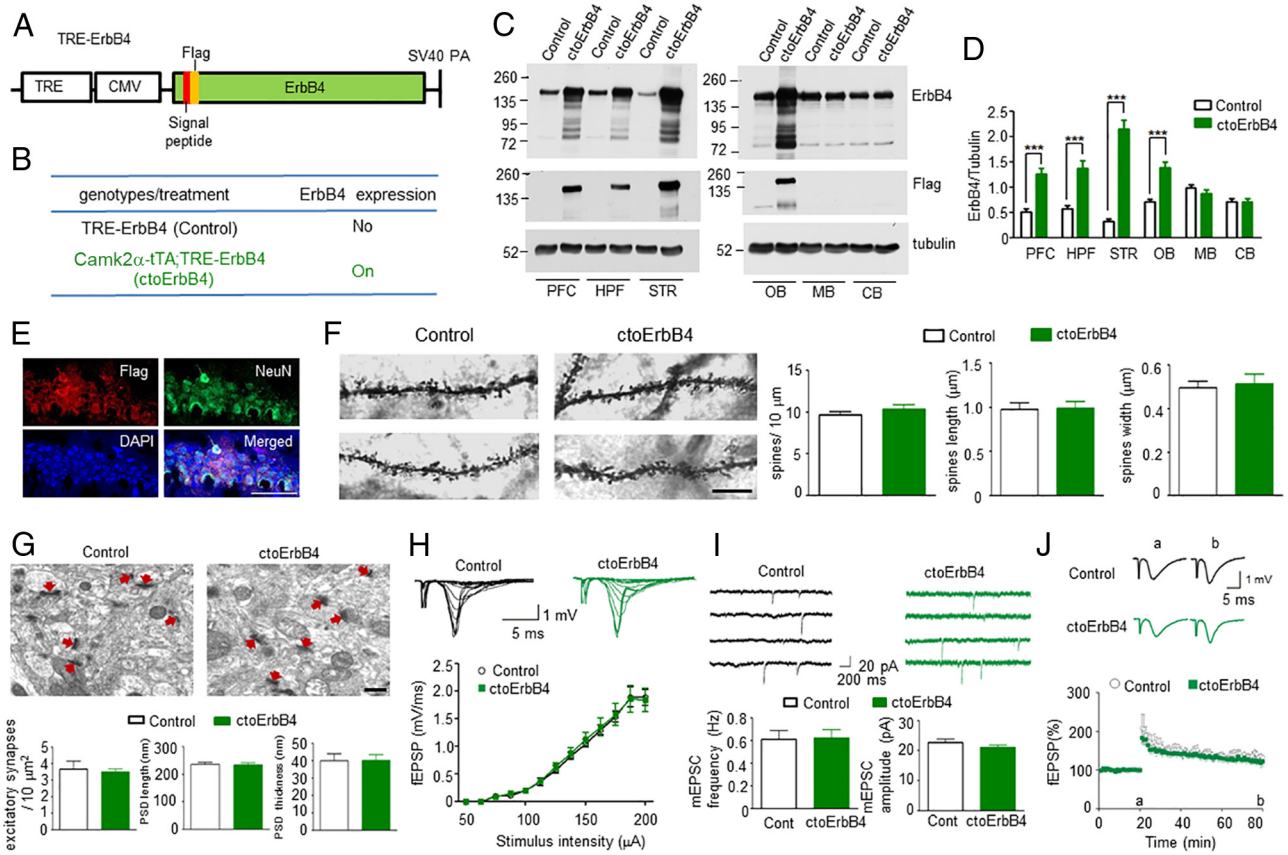


**Figure 2.** Overexpression of ErbB4 in pyramidal neurons did not alter spine formation and excitatory synaptic transmission *in vivo*. ***A***, TRE-*ErbB4* transgene structure. Full-length *ErbB4* was cloned into pMM400 after the promoter complex of TRE and CMV (cytomegalus virus minimal promoter), before the SV40 polyadenylation signal. ***B***, Genotypes and ErbB4 expression. Blank and green histograms/curves represent data from control and cto*ErbB4* mice, respectively. ***C***, Expression of ErbB4 transgene in prefrontal cortex (PFC), hippocampus (HPF), striatum (STR), and olfactory bulb (OB), but not midbrain (MB) and cerebellum (CB). Homogenates of various brain regions were subjected to Western blot analysis with indicated antibodies. ***D***, Quantification of ErbB4 expression in different brain regions. Expression levels of ErbB4 were normalized by tubulin. $n = 3$ per genotype. $^{***}p < 0.001$ for PFC, HPF, STR, and OB (one-way ANOVA). ***E***, Flag staining in the hippocampal CA1 region of cto*ErbB4* mice. Sections were tripled-stained with anti-Flag antibody, anti-NeuN antibody, and DAPI. Scale bar, 50 $\mu$m. ***F***, The density, length, and width of spines were indistinguishable in the CA1 region between control and cto*ErbB4* mice. Left, Representative Golgi staining images. Right, Quantification data. $n = 34$ neurons from 3 mice per group. Scale bar, 10 $\mu$m. ***G***, Comparable numbers of excitatory synapses, PSD length, and PSD thickness in the CA1 region between control and cto*ErbB4* mice. Top, Representative EM images. Bottom, Quantification data. $n = 105$ images from 3 mice per group. Arrows indicate PSD. Scale bar, 0.5 $\mu$m. ***H***, Similar I/O curves at SC–CA1 synapses between control and cto*ErbB4* mice. Top, Representative traces of fEPSPs. Bottom, I/O curves. $n = 10$ slices from 4 mice per group. ***I***, No difference in mEPSC frequency and amplitude between control and cto*ErbB4* mice. Top, Representative traces. Bottom, Quantitative data. $n = 9$ cells from 3 mice per group. ***J***, Similar LTP at SC–CA1 synapses between control and cto*ErbB4* mice. Top, Representative traces. Bottom, Quantification data. $n = 6$ slices from 4 mice per group.

LTP was induced by theta-burst stimulation, 10 trains of 4 pulses at 100 Hz with an interval of 200 ms.

*Western blot.* Brain tissues were homogenized in RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% SDS, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM DTT, and protease inhibitor cocktails. Homogenates were resolved on SDS-PAGE and transferred to nitrocellulose membranes, which were incubated in the TBS containing 0.1% Tween 20 and 5% milk for 1 h at room temperature before the addition of primary antibody for incubation overnight at 4°C. After wash, membranes were incubated with HRP-conjugated secondary antibody in the TBS buffer for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce). Films were scanned with an Epson 1680 scanner and analyzed with ImageJ (National Institutes of Health). The following primary antibodies were used: rabbit anti-ErbB4 (0618, 1:2000, generously provided by Dr. Cary Lai), rabbit anti-Flag (1:1000, Sigma), and mouse anti-$\alpha$-tubulin (1:5000, Cell Signaling Technology).

*Immunofluorescence.* Anesthetized mice were perfused transcardially with 4% PFA in PBS, and tissues were fixed in 4% PFA at 4°C for 5 h. Frozen brain blocks were cut into 40-$\mu$m-thick sections on a vibrating microtome (VT1000S; Leica Microsystem). Sections were incubated with 0.3% Triton X-100 and 5% BSA in PBS at room temperature for 1 h. Slices were then incubated with mouse anti-NeuN (1:1000, Millipore) or rabbit anti-Flag (1:1000, Sigma) antibodies at 4°C overnight. After washing with PBS for 3 times, samples were incubated with AlexaFluor-conjugated secondary antibodies (1:1000, Invitrogen) for 1 h at room temperature. Samples were mounted with Vectashield mounting medium (Vector), and images were taken by Zeiss LSM510 confocal microscope.

*Statistics analysis.* Data from Golgi staining and electron microscopic analysis and mEPSCs were analyzed by Student's *t* test. One-way ANOVA was used to analyze data from Western blot in cto*ErbB4* mice. Student's *t* test was used to analyze Western blot data from Camk2$\alpha$-*ErbB4*$^{-/-}$ and PV-*ErbB4*$^{-/-}$ mice. Two-way ANOVA was performed for spine densities at different ages and for data of input–output (I/O) curve and paired pulse facilitation (PPF). Data were expressed as mean ± SEM unless otherwise indicated.

## Results

### ErbB4 in pyramidal neurons is dispensable for spine formation

To determine whether ErbB4 in pyramidal neurons is important for spine and excitatory synapse formation, we generated excitatory neuron-specific ErbB4 mutant mice by crossing *ErbB4*f/f mice with Camk2$\alpha$::Cre mice that express Cre in forebrain pyramidal neurons (Fig. 1*A*) (Tsien et al., 1996). ErbB4 levels in the

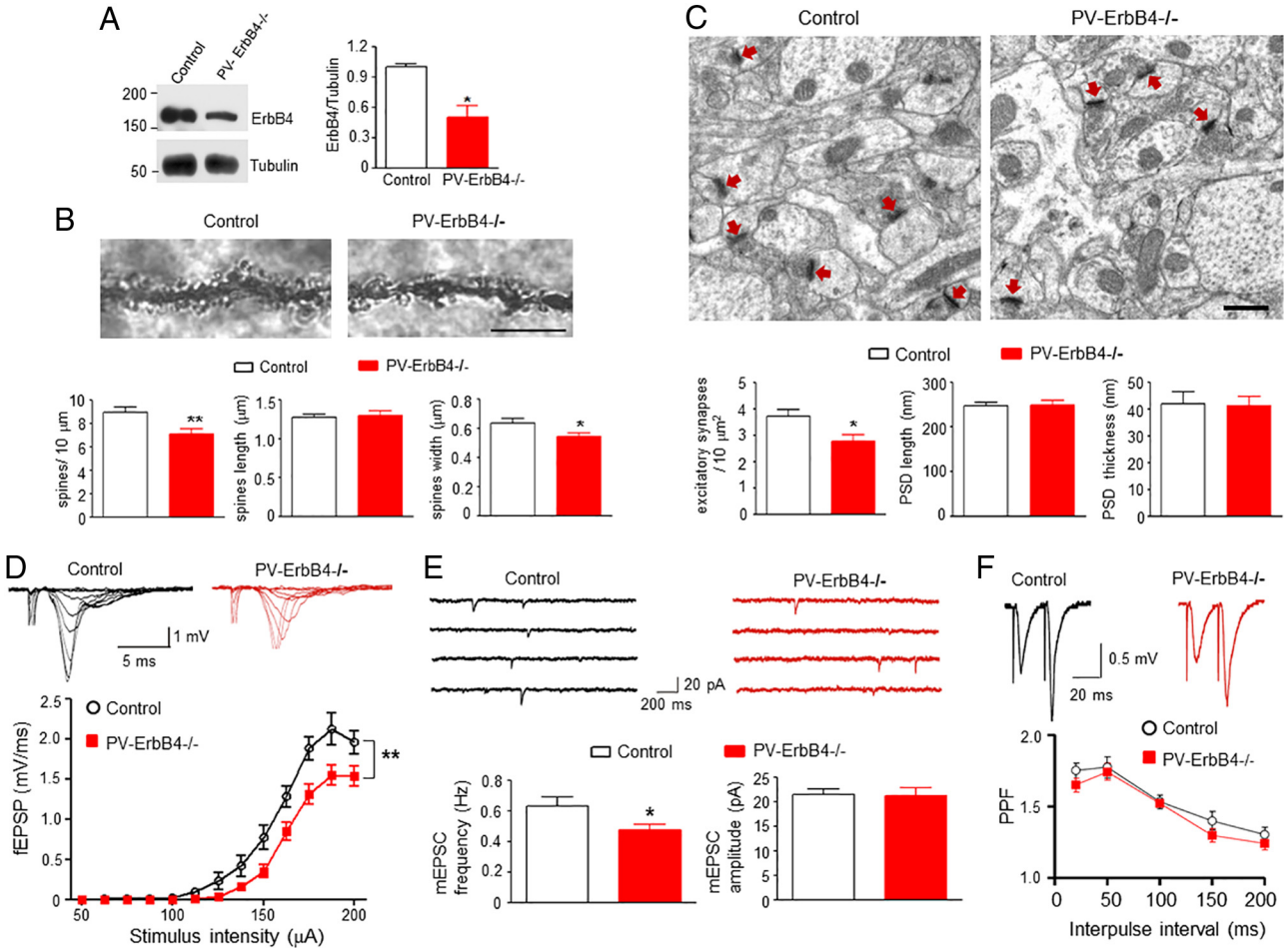


**Figure 3.** Deletion of ErbB4 from PV-positive neurons impairs spine formation. ***A***, Reduction of ErbB4 protein levels in the hippocampus of PV-*ErbB4* $^{-/-}$ mice. Hippocampal homogenates of 2-month-old mice were subjected to Western blotting. Left, Representative blots of ErbB4 and tubulin. Right, Quantification data. Expression levels of ErbB4 were normalized by tubulin. $n = 3$ mice per group. $^{*}p < 0.05$ (*t* test). ***B***, Reduced spine density and width in the CA1 region of PV-*ErbB4* $^{-/-}$ mice. Top, Representative Golgi staining images. Bottom, Quantitative data. $n = 68$ neurons from 6 mice per group. $^{**}p < 0.01$ (*t* test). Scale bar, 10 μm. ***C***, Fewer excitatory synapses in PV-*ErbB4* $^{-/-}$ mice compared with control littermates. PSD length and thickness did not show significant difference between the two groups. Top, Representative EM images from CA1 regions. Bottom, Quantitative data. $n = 108$ images from 3 mice per group. $^{*}p < 0.05$ (*t* test). Arrows indicate PSD. Scale bar, 0.5 μm. ***D***, Impaired I/O curves at SC–CA1 synapses in PV-*ErbB4* $^{-/-}$ mice. Top, Representative traces of fEPSPs. Bottom, I/O curves. $n = 14$ slices from 6 mice per group. $^{**}p < 0.01$ (two-way ANOVA). ***E***, Reduced mEPSC frequency in PV-*ErbB4* $^{-/-}$ mice. Top, Representative mEPSC traces. Bottom, Quantitative data. $n = 12$ cells from 4 mice per group. $^{*}p < 0.05$ (*t* test). ***F***, Normal PPF in PV-*ErbB4* $^{-/-}$ hippocampal slices. Top, Representative fEPSP traces. Bottom, Quantitative data. $n = 12$ slices from 4 mice per group.

hippocampus in Camk2α::Cre;*ErbB4*f/f mice (thereafter referred as Camk2α-*ErbB4* $^{-/-}$ mice) was not changed significantly, compared with control mice (Fig. 1*B*) (Chen et al., 2010). This result suggests low-level (if any) of ErbB4 expression in pyramidal neurons, in agreement with previous reports (Yau et al., 2003; Vullhorst et al., 2009; Fazzari et al., 2010). Golgi staining analysis indicated that the spine density of CA1 pyramidal neurons was comparable between Camk2α-*ErbB4* $^{-/-}$ mice and control littermates (Fig. 1*C*). No difference was observed in spine length and width between the two genotypes (Fig. 1*C*). These results suggest that the number and morphology of spines may not be altered by ErbB4 deletion in CA1 pyramidal neurons. We further analyzed the number of excitatory synapses in the CA1 region by transmission electron microscopy (EM). As shown in Figure 1*D*, similar numbers of excitatory synapses were observed between Camk2α-*ErbB4* $^{-/-}$ mice and littermate controls. The length and thickness of PSD were also similar between the two genotypes (Fig. 1*D*). These results indicate that ErbB4 in pyramidal neurons is not critical for the formation of spines and excitatory synapses. They extend previous findings that genetic ablation of ErbB4 from pyramidal neurons by viral expression of Cre does not alter the density or function of dendritic spines (Fazzari et al., 2010).

To determine whether ErbB4 in pyramidal neurons is important for excitatory synaptic transmission, we measured glutamate transmission at SC–CA1 synapses. The I/O curves of fEPSPs represent basal AMPA receptor-mediated currents and were comparable between Camk2α-*ErbB4* $^{-/-}$ mice and control littermates (Fig. 1*E*). Moreover, the frequency and amplitude of mEPSCs were also similar between the two genotypes (Fig. 1*F*). These observations, together with the morphological results, suggest that ErbB4 in CA1 pyramidal neurons is dispensable for the structure and function of dendritic spines.

### Overexpression of ErbB4 in pyramidal neurons does not affect spine formation

Next, we asked whether the increase of ErbB4 in pyramidal neurons *in vivo* could affect spine formation and maturation. To this end, TRE-*ErbB4* mice were generated that carry the human *ErbB4* (JMa, CYT2 isoform) cDNA under the control of the tetracycline-responsive promoter element (TRE) tetO (Fig.

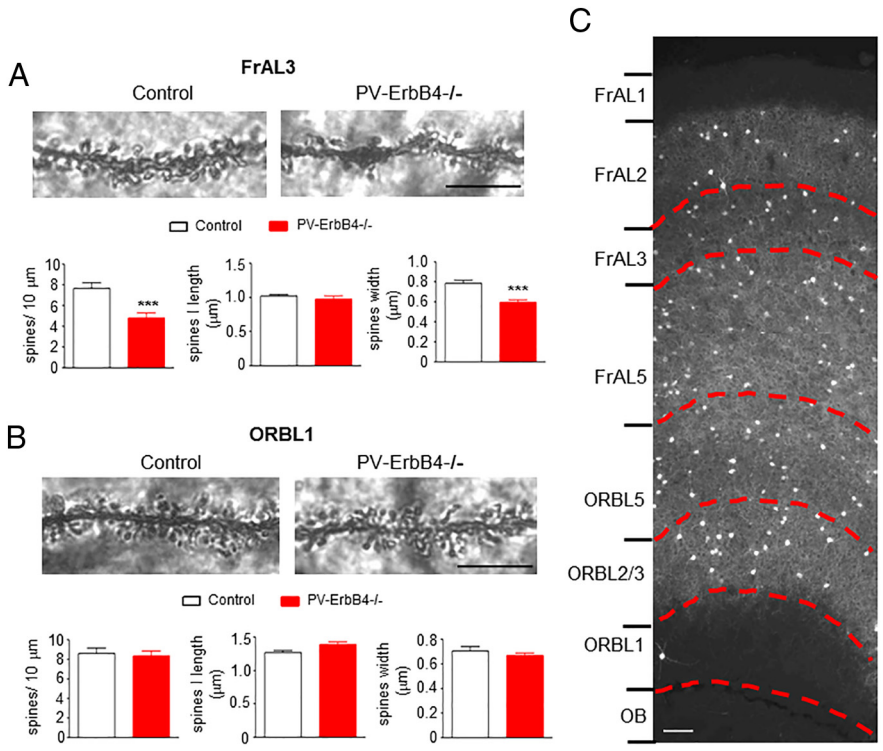


**Figure 4.** Reduction of spine density in FrAL3 of PV-*ErbB4* $^{-/-}$ mice. ***A***, Reduced spine density and width in FrAL3 of PV-*ErbB4* $^{-/-}$ mice. Top, Representative Golgi staining images of FrAL3 pyramidal neurons. Bottom, Quantitative data. $n = 32$ neurons from 3 mice per group. ***$p < 0.001$ (*t* test). Scale bar, 10 $\mu$m. ***B***, Normal spine density and width in ORBL1 of PV-*ErbB4* $^{-/-}$ mice. Top, Representative Golgi staining images of ORBL1 pyramidal neurons. Bottom, Quantitative data. $n = 34$ neurons from 3 mice per group. Scale bar, 10 $\mu$m. ***C***, Fewer PV-positive cells in ORBL1 of the PFC. Cortical slices were stained with anti-PV antibody, which was visualized by AlexaFluor-conjugated secondary antibodies. OB, Olfactory bulb. Scale bar, 100 $\mu$m.

2*A*). A Flag tag was inserted immediately after the artificial signal peptide of *ErbB4* cDNA, which did not interfere with ErbB4 processing and function (Huang et al., 2000). TRE-*ErbB4* mice did not express ErbB4 in the absence of tetracycline transactivator (tTA) (Fig. 2*B*,*C*). To overexpress ErbB4 specifically in pyramidal neurons, TRE-*ErbB4* mice were crossed with Camk2$\alpha$::tTA mice that express tTA in excitatory neurons (Mayford et al., 1996). Resulting bitransgenic Camk2$\alpha$::tTA;TRE-*ErbB4* mice (named as cto*ErbB4* mice for *C*amk2$\alpha$ promoter-driven *t*et-*o*ff *ErbB4*) produced Flag-ErbB4 in excitatory neurons (Fig. 2*C*–*E*). Compared with TRE-*ErbB4* or control mice, ErbB4 was increased by onefold to twofold in the prefrontal cortex (PFC) and hippocampus in cto*ErbB4* mice (Fig. 2*C*,*D*). The increase was the result of expression of the transgene, which was detectable by an anti-Flag antibody (Fig. 2*C*,*E*). Moreover, the increase was forebrain-specific, and not detectable in the midbrain and cerebellum (Fig. 2*C*,*D*), in agreement with the expression of Camk2$\alpha$ tTA (Mayford et al., 1996). Together, these results indicate that cto*ErbB4* mice express higher levels of ErbB4 in pyramidal neurons in forebrain regions, including hippocampus.

As shown in Figure 2*F*, the density, length, and width of spines in CA1 pyramidal neurons were comparable between cto*ErbB4* mice and control littermates, suggesting that overexpression of ErbB4 in pyramidal neurons does not alter the number or size of dendritic spines. To further test this hypothesis, we performed two additional experiments. First, we examined excitatory synapses in the CA1 region by EM. The number of excitatory synapses and the PSD length and thickness were indistinguishable between cto*ErbB4* mice and control littermates (Fig. 2*G*), in agreement with studies from Golgi staining. Second, we characterized glutamate transmission at SC–CA1 synapses. As shown in Figure 2*H*, I/O curves of fEPSPs were similar between cto*ErbB4* mice and control littermates, suggesting that *in vivo* overexpression of ErbB4 in pyramidal neurons did not alter basal glutamatergic transmission. In agreement, the frequency and amplitude of mEPSCs were also comparable between the two genotypes (Fig. 2*I*). Moreover, LTP at SC–CA1 synapses was similar between cto*ErbB4* and control hippocampal slices (Fig. 2*J*). These findings were consistent with previous reports that ErbB4 in pyramidal neurons is not crucial for NRG1 to suppress LTP (Chen et al., 2010; Shamir et al., 2012). Together, these observations indicate that increased ErbB4 levels in excitatory neurons do not alter the number, structure, or function of excitatory synapses in pyramidal neurons.

### ErbB4 deletion in PV-positive GABAergic interneurons impairs spine formation

Spine density in hippocampal pyramidal neurons was reduced by ErbB4 deletion in all neural cells (Barros et al., 2009). We demonstrate that ErbB4 ablation or overexpression in excitatory neurons has little effect on the number and function of excitatory synapses in the hippocampus (Figs. 1 and 2). These observations suggest that ErbB4 in nonexcitatory neurons may regulate spine formation or function. Given that ErbB4 is highly expressed in PV-positive GABAergic interneurons (Yau et al., 2003; Vullhorst et al., 2009; Fazzari et al., 2010), we speculated that spine impairment may be mediated by ErbB4 deficiency in PV-positive neurons. To test this hypothesis, we crossed *ErbB4*f/f mice with PV::Cre mice to get the PV::Cre;*ErbB4*f/f mice (referred to as PV-*ErbB4* $^{-/-}$ mice thereafter). Unless otherwise indicated, mice at 2 months of age were characterized for ErbB4 expression, spine morphology, and electrophysiology. ErbB4 was specifically deleted from PV-positive neurons in PV-*ErbB4* $^{-/-}$ mice (Chen et al., 2010; Wen et al., 2010; Shamir et al., 2012). As shown in Figure 3*A*, ErbB4 protein in hippocampus was significantly decreased in PV-*ErbB4* $^{-/-}$ mice compared with control mice, suggesting that ErbB4 is largely restricted into GABAergic interneurons (Yau et al., 2003; Vullhorst et al., 2009; Fazzari et al., 2010). Intriguingly, spine density and width of CA1 pyramidal neurons were reduced in PV-*ErbB4* $^{-/-}$ mice compared with control littermates (Fig. 3*B*). EM analysis demonstrated fewer excitatory synapses in hippocampal CA1 region in PV-*ErbB4* $^{-/-}$ mice than control littermates (Fig. 3*C*).

In accordance with the structural changes, I/O curves of fEPSPs were shifted downward in PV-*ErbB4* $^{-/-}$ hippocampal slices compared with control littermates (Fig. 3*D*), suggesting impaired basal glutamatergic transmission at SC–CA1 synapses. In addition, the frequency, but not the amplitude, of mEPSCs was reduced in PV-*ErbB4* $^{-/-}$ mice compared with their control littermates (Fig. 3*E*). To determine whether decreased mEPSC frequency results from reduced number of functional synapses or impaired glutamate release, we measured fEPSPs evoked by two

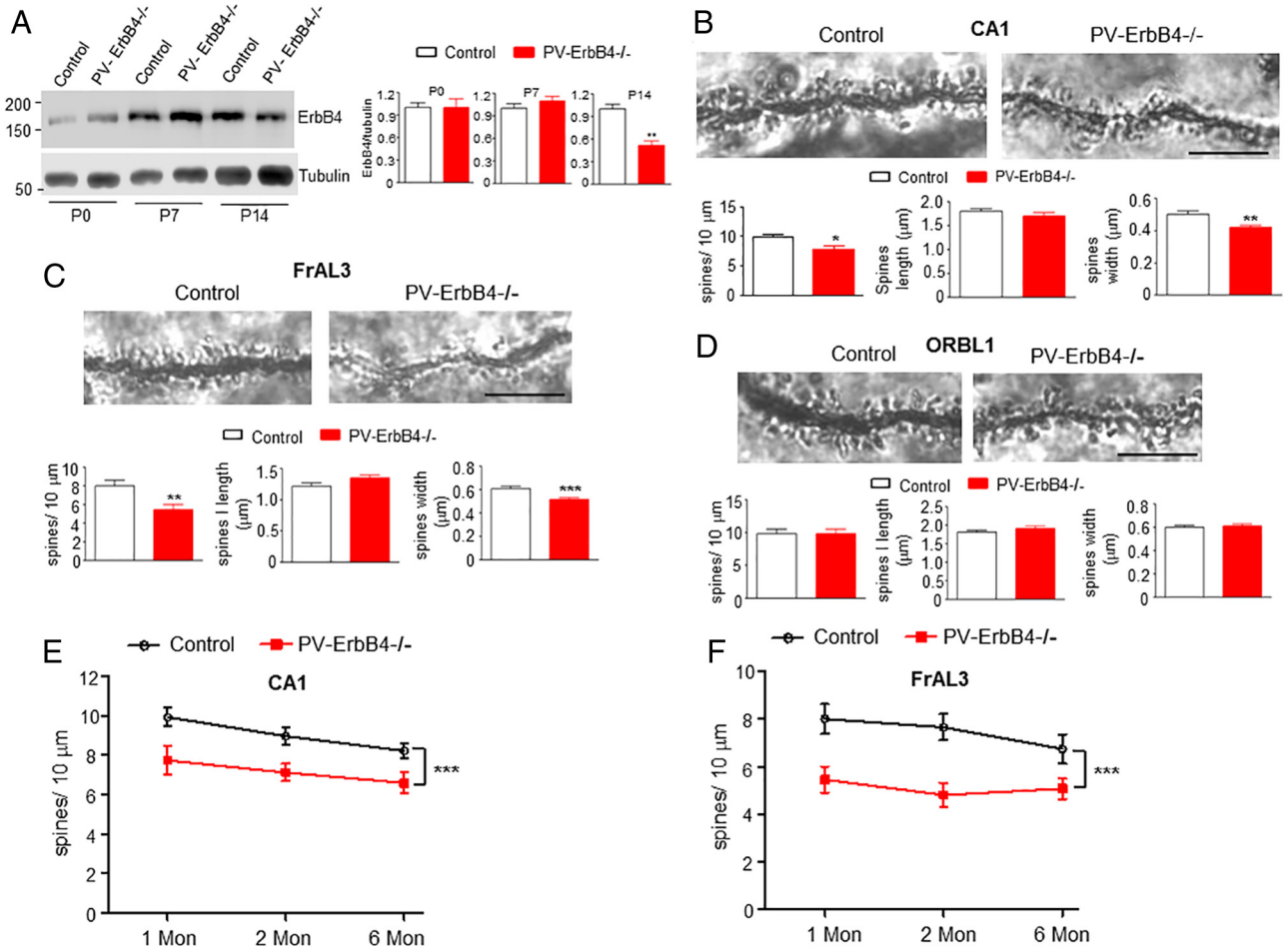


**Figure 5.** Impaired spine formation, but not degeneration, in PV-*ErbB4*$^{-/-}$ mice. ***A***, ErbB4 was decreased in PV-*ErbB4*$^{-/-}$ hippocampus at P14. Left, Representative blots of ErbB4 and tubulin from P0, P7, or P14 control and PV-*ErbB4*$^{-/-}$ mice. Right, Quantification data. Expression levels of ErbB4 were normalized by tubulin. $n = 3$ mice per group. $^{**}p < 0.01$ (*t* test). ***B***, Reduced spine density and width in the CA1 region of 1-month-old PV-*ErbB4*$^{-/-}$ mice. Top, Representative Golgi staining images of CA1 pyramidal neurons. Bottom, Quantitative data. $n = 30$ neurons from 3 mice per group. $^{**}p < 0.01$ (*t* test). $^{*}p < 0.05$ (*t* test). Scale bar, 10 $\mu$m. ***C***, Reduced spine density and width in FrAL3 of 1-month-old PV-*ErbB4*$^{-/-}$ mice. Top, Representative Golgi staining images of FrAL3 pyramidal neurons. Bottom, Quantitative data. $n = 33$ neurons from 3 mice per group. $^{***}p < 0.001$ (*t* test). $^{**}p < 0.01$ (*t* test). Scale bar, 10 $\mu$m. ***D***, Normal spine density and width in ORBL1 of 1-month-old PV-*ErbB4*$^{-/-}$ mice. Top, Representative Golgi staining images of ORBL1 pyramidal neurons. Bottom, Quantitative data. $n = 32$ neurons from 3 mice per group. Scale bar, 10 $\mu$m. ***E***, Spine density in the CA1 region of PV-*ErbB4*$^{-/-}$ and control mice at different ages. $^{***}p < 0.001$ (two-way ANOVA). ***F***, Spine density in FrAL3 of PV-*ErbB4*$^{-/-}$ and control mice at different ages. $^{***}p < 0.001$ (two-way ANOVA).

presynaptic stimulations delivered at different intervals (i.e., paired pulses). The PPF of initial slopes of fEPSPs was comparable between PV-*ErbB4*$^{-/-}$ and control slices (Fig. 3*F*), indicating no alteration of glutamate release. These results suggest that reduced mEPSC frequency in PV-*ErbB4*$^{-/-}$ mice is likely the result of less excitatory synapses. Together, these results demonstrate that ErbB4 in PV-positive interneurons plays a critical role in regulating excitatory synapse formation in pyramidal neurons.

### Reduced spine density in PFC layer 3 in PV-*ErbB4*$^{-/-}$ mice

PFC is increasingly implicated in schizophrenia. In particular, spine density reduction was observed in the PFC of patients with schizophrenia (Glantz and Lewis, 2000). Having demonstrated a role of ErbB4 in regulating spine density in the hippocampus, we next determined whether spine formation in the PFC is regulated by similar mechanisms. To this end, we analyzed dendritic spines in neurons in superficial and deep layers of PFC. As shown in Figure 4*A*, spine density and width, revealed by Golgi staining, were reduced in the frontal association area layer 3 (FrAL3) of PFC in PV-*ErbB4*$^{-/-}$ mice, compared with control littermates. However, spine density and width in orbital area layer 1 (ORBL1) appeared not altered by ErbB4 deletion in PV-positive neurons (Fig. 4*B*). This regional difference of ErbB4 ablation effect on spines may be the result of fewer PV-positive neurons in ORBL1, compared with FrAL3 (Fig. 4*C*).

### Reduction of spine density in adolescent PV-*ErbB4*$^{-/-}$ mice

To determine whether spine density reduction in 2-month-old PV-*ErbB4*$^{-/-}$ mice was the result of developmental deficit or degeneration, we determined when the ErbB4 protein was decreased in PV-*ErbB4*$^{-/-}$ mice. As shown in Figure 5*A*, ErbB4 reduction was not apparent in early postnatal days (P0 and P7); however, significant reduction was observed in the hippocampus in P14 PV-*ErbB4*$^{-/-}$ mice. This time course was consistent with that of PV promoter activity during development (del Río et al., 1994). Given that spine formation peaks in postnatal weeks 3–5 (Boyer et al., 1998), we next determined whether spine density was reduced during this stage in PV-*ErbB4*$^{-/-}$ mice. Golgi staining was performed to analyze neurons in hippocampus and PFC from 1-month-old mice. Intriguingly, the reduction in spine density and width was observed in the PV-*ErbB4*$^{-/-}$ CA1 region and FrAL3 in 1 month of age, compared with control littermates

(Fig. 5*B*,*C*). In contrast, the reduction of spine density and width did not occur in the ORBL1 from 1-month-old PV-*ErbB4*$^{-/-}$ mice (Fig. 5*D*), in agreement with the results from 2-month-old PV-*ErbB4*$^{-/-}$ mice (Fig. 4*B*). To address whether the spine reduction in PV-*ErbB4*$^{-/-}$ mice evolves with aging, we analyzed dendritic spines in 6-month-old PV-*ErbB4*$^{-/-}$ mice and control littermates. Spine reduction in PV-*ErbB4*$^{-/-}$ mice did not worsen with aging, compared with control littermates (Fig. 5*E*,*F*). These results demonstrate that the reduction of spine density in PV-*ErbB4*$^{-/-}$ mice may result from impaired spine formation, not neuronal degeneration.

## Discussion

ErbB4 has been implicated in the formation of excitatory synapses in the hippocampus and cortex. Conditional ablation of ErbB2 and ErbB4 (generated by hGFAP::Cre) reduces dendritic spine density in hippocampal and cortical neurons in single- and double-mutant mice (Barros et al., 2009). The cellular mechanisms by which ErbB4 regulates spine formation remain unclear. ErbB4 knockdown from pyramidal neurons reduces spine density and size in organotypical hippocampal slices, suggesting that ErbB4 may be necessary for spine formation (Li et al., 2007). On the other, ablation of ErbB4 in pyramidal neurons of floxed-*ErbB4* mice (using retroviral Cre expression) did not alter the density of dendritic spines or mEPSC (Fazzari et al., 2010). In light of accumulating evidence that ErbB4 expression is high or specific in GABAergic interneurons and low or absent in pyramidal neurons (Huang et al., 2000; Yau et al., 2003; Vullhorst et al., 2009; Fazzari et al., 2010), we mutated the *ErbB4* gene specifically in PV-positive cells, a major type of interneurons that express ErbB4 (Vullhorst et al., 2009; Fazzari et al., 2010), and pyramidal neurons and determined the *in vivo* effect of *ErbB4* mutation on spine development. We found that ablation of the *ErbB4* gene (by Camk2$\alpha$::Cre) from pyramidal neurons did not affect spine density or function in the hippocampus and cortex. In contrast, the spine density was reduced in PV-*ErbB4*$^{-/-}$ mice where ErbB4 was selectively deleted from PV-positive GABAergic interneurons. These observations indicate that ErbB4 in PV-positive neurons promotes spine formation during development. In support of our hypothesis is a recent finding that demonstrated a non–cell-autonomous role for ErbB4 in spine formation (Del Pino et al., 2013).

Because hGFAP::Cre expresses in neural precursors starting at E13.5 (Zhuo et al., 2001), a parsimonious model is that spine deficits in the conditional ErbB2/ErbB4 double-mutant mice (Barros et al., 2009) are the result of defects in nonpyramidal cells. Loss of ErbB4 in interneurons compromises GABAergic transmission (Woo et al., 2007; Chen et al., 2010; Wen et al., 2010). During early development, GABA depolarizes immature neurons resulting from high intracellular $Cl^-$ concentration (Ben-Ari, 2002; Owens and Kriegstein, 2002). GABA-mediated depolarization is critical in the formation of spines and excitatory synapses (Ben-Ari et al., 1997; Akerman and Cline, 2006; Ge et al., 2006; Wang and Kriegstein, 2008). Thus, in ErbB4 mutant mice, impaired GABA release from interneurons during early development might lead to fewer spines and excitatory synapses. In support of this hypothesis was the observation that ErbB4 protein is reduced in PV-*ErbB4*$^{-/-}$ mice at postnatal day 14, before the peak of spine formation (Boyer et al., 1998).

Alternatively and perhaps in addition, deletion of ErbB4 from fast-spiking interneurons (which are PV-positive) impairs the assembly and function of the GABAergic circuitry (Fazzari et al., 2010; Ting et al., 2011; Del Pino et al., 2013). Compromised GABA transmission in ErbB4 mutant mice increases the activity of pyramidal neurons (Wen et al., 2010; Del Pino et al., 2013), which may lead to a compensatory reduction in spine formation. Consistent with this notion, in PV-*ErbB4*$^{-/-}$ mice, spine reduction was observed in hippocampal CA1 and FrAL3 of the PFC, where PV-positive cells were abundant, but not in ORBL1 of the PFC, where few PV-positive cells were present (Fig. 4). Moreover, ErbB4 in PV-positive GABAergic interneurons, but not in pyramidal neurons, is crucial for NRG1 regulation of synaptic plasticity, such as LTP (Chen et al., 2010; Shamir et al., 2012). Together, these studies provide strong evidence that ErbB4 in GABAergic interneurons plays a role in the development and function of cortical and hippocampal excitatory synapses.

Acute overexpression of ErbB4 in pyramidal neurons was shown to increase spine size but not density in organotypical hippocampal slices (Li et al., 2007). In cto*ErbB4* mice, the spine size and excitatory synaptic transmission in the hippocampus were similar to those of control mice (Fig. 2), suggesting that increasing ErbB4 expression in pyramidal neurons is not able to alter spines *in vivo*. We notice the difference of levels and duration of ErbB4 expression between the two studies. ErbB4 expression by transient *in vitro* transfection might be higher than that in cto*ErbB4* mice. Spines were analyzed in 2-month-old cto*ErbB4* mice when synapses are pretty mature, whereas spines may be immature in DIV 6–9 hippocampal slices (2–3 d after transfection) (Li et al., 2007). Homeostatic mechanisms may have reduced the effect of ErbB4 overexpression in cto*ErbB4* mice.

The spine density is reduced in the PFC and hippocampus of schizophrenia patients (Garey et al., 1998; Glantz and Lewis, 2000; Kolomeets et al., 2005), which may contribute to impaired glutamatergic transmission in schizophrenia (Lewis and Sweet, 2009; Yin et al., 2012). Interestingly, spine reduction is more dramatic in layer 3 PFC than in other brain regions in patients with schizophrenia (Glantz and Lewis, 2000) and in PV-*ErbB4*$^{-/-}$ mice (Fig. 4). Reduced NRG1 protein levels and ErbB4 truncation mutants have been reported in patients with schizophrenia (Bertram et al., 2007; Walsh et al., 2008; Parlapani et al., 2010). Thus, compromised spine density may serve as a pathophysiological mechanism of schizophrenia related with NRG1 and ErbB4.

Although the majority of spines are localized on dendrites of pyramidal neurons, spines are detectable on dendrites of some PV-negative GABAergic interneurons, including somatostatin- and calbindin-positive cells (Guirado et al., 2013). ErbB4 expression appears to be low in somatostatin- and calbindin-positive GABAergic interneurons in hippocampus (Neddens and Buonanno, 2010). However, some somatostatin-positive cells in cerebral cortex express ErbB4 (Yau et al., 2003). Given that ErbB4 is enriched in postsynaptic fraction and interacts with PSD95 (Garcia et al., 2000; Huang et al., 2000), it would be interesting to determine whether ErbB4 may also regulate the spine formation in somatostatin-positive cells in cerebral cortex.

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