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Ablation of ErbB4 from excitatory neurons leads to reduced dendritic spine density in mouse prefrontal cortex

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Abstract

Dendritic spine loss is observed in many psychiatric disorders, including schizophrenia, and likely contributes to the altered sense of reality, disruption of working memory, and attention deficits that characterize these disorders. ErbB4, a member of the EGF family of receptor tyrosine kinases, is genetically associated with schizophrenia, suggesting that alterations in ErbB4 function contribute to the disease pathology. Additionally, ErbB4 functions in synaptic plasticity, leading us to hypothesize that disruption of ErbB4 signaling may affect dendritic spine development. We show that dendritic spine density is reduced in the dorsomedial prefrontal cortex of ErbB4 conditional whole-brain knockout mice. We find that ErbB4 localizes to dendritic spines of excitatory neurons in cortical neuronal cultures and is present in synaptic plasma membrane preparations. Finally, we demonstrate that selective ablation of ErbB4 from excitatory neurons leads to a decrease in the proportion of mature spines and an overall reduction in dendritic spine density in the prefrontal cortex of weanling (P21) mice that persists at 2 months of age. These results suggest that ErbB4 signaling in excitatory pyramidal cells is critical for the proper formation and maintenance of dendritic spines in excitatory pyramidal cells.

Keywords

schizophrenia; neuregulin; receptor tyrosine kinase; layer V; neurodevelopment;
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RRID:MGI_MGI:4429427; RRID:AB_2099883; RRID:AB_94235; RRID:AB_2140110;
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INTRODUCTION

Schizophrenia is a debilitating psychiatric disorder that typically strikes in early adulthood, likely due to a disturbance of synaptic maturation or stabilization processes that normally

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ROLE OF AUTHORS

M.A.C conceived and performed experiments and wrote the manuscript. A.J.K. helped conceive experiments and edited the manuscript.

occur in dendritic spines during adolescence (Weinberger, 1987). Indeed, postmortem examinations from the brains of schizophrenia patients reveal a reduction in cortical thickness that results from a loss of dendrites and dendritic spines (Glantz and Lewis, 2000; Glausier and Lewis, 2012; Selemon et al., 2002). The failure to form and maintain dendritic spines ultimately leads to the reduction of synaptic connections, contributing to the disruption of normal perception, cognition, affect, and motor skills in individuals affected by schizophrenia (Lewis, 2009; Selemon and Goldman-Rakic, 1999). Thus, a comprehensive understanding of the cellular and molecular mechanisms that control synapse development and dendritic spine stability should lead to better strategies to treat and prevent schizophrenia.

Genetic susceptibility and environmental stressors are both major risk factors for schizophrenia. Schizophrenia is highly heritable: direct offspring of individuals with schizophrenia have increased risk of developing the disease, which rises to as much as 50% in identical twins (Gottesman, 1991). Among the susceptibility genes associated with schizophrenia, Neuregulin 1 (Nrg1), the primary ligand for the receptor tyrosine kinase, ErbB4, has been identified in several studies (Addington et al., 2007; Law et al., 2006; Mei and Xiong, 2008; Stefansson et al., 2002). These findings raise the question of how alterations in ErbB4 signaling might contribute to the pathophysiological hallmarks of schizophrenia.

ErbB4 is highly expressed in the brain, and both its mRNA and protein are elevated in affected areas of the schizophrenic brain, including the hippocampus and dorsal prefrontal cortex (Chong et al., 2008; Silberberg et al., 2006) where it functions in synapse maturation and plasticity (Krivosheya et al., 2008; Li et al., 2007a; Mei and Xiong, 2008). In both hippocampal slice and dissociated neuron cultures, activation of ErbB4 signaling by elevated receptor levels or treatment with its ligand, Nrg1, promotes the enlargement of synaptic contacts and drives more activated ErbB4 into the postsynaptic density (PSD) (Barros et al., 2009; Gu et al., 2005; Li et al., 2007a; Ma et al., 2003). However, where ErbB4 acts to control spine and synapse density is an unresolved issue. ErbB4 is abundantly expressed in GABAergic interneurons, and it has been proposed that disruption of inhibitory inputs onto excitatory pyramidal neurons may contribute to the changes in dendritic spine numbers in ErbB4 mutant mice (Wen et al., 2010; Yin et al., 2013). Therefore, whether and how the loss of ErbB4 function in excitatory neurons in vivo affects dendritic spine development or maintenance is still unclear.

We provide evidence here that ErbB4 function is required in excitatory neurons for proper dendritic spine development in the prefrontal cortex. We find that ErbB4 protein is present in excitatory neurons in the cortex. Additionally, ErbB4 is enriched in purified synaptoneurosome preparations and an ErbB4-GFP fusion protein localizes to dendritic spines in cultured cortical neurons. We demonstrate that selective inactivation of ErbB4 in excitatory neurons leads to reductions in spine density and overall changes in spine morphology in select dendritic regions of layer V pyramidal neurons that persist into adulthood. Interestingly, the layer-specific reductions in spine density in these ErbB4-deficient mice resemble the layer-specific losses of dendritic spines observed in post-mortem analysis of schizophrenia patients (Black et al., 2004; Glantz and Lewis, 2000). Our

data suggest that perturbations in ErbB4 signaling in excitatory neurons may contribute to the reduced dendritic spine density observed in patients with schizophrenia.

MATERIALS AND METHODS

Animals

Mice were housed and cared for in accordance to the Yale University Institutional Care and Use Committee. Frozen embryos (RRID:IMSR_MMRRC:010439, Stock #: 010439-UCD) harboring a “floxed” *erbb4* allele were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at UC Davis (Golub et al., 2004). These embryos were implanted in pseudopregnant foster mothers by the Yale Animal Genomics Services and the resulting pups were genotyped with the following primers as per the MMRRC instructions: Primer 1 – 5' CAAATGCTCTCTCTGTTCTTTGTGTCTG, Primer 2 – 5' TTTTGCCAAGTTCTAATTCATCAGAAGC, Primer 3 – 5' TATTGTGTTCATCTATCATTGCAACCCAG. Mice expressing two copies of the floxed *erbb4* allele were then crossed with the *thy1*-GFP M1 line, RRID:IMSR_JAX:007788 (Feng et al., 2000) and either Nestin-Cre, RRID:MGI_MGI:3840982 (Isaka et al., 1999), which is expressed homogenously throughout the forebrain after embryonic day 10.5 or Nex-Cre, RRID:MGI_MGI:4429427 (Fazzari et al., 2010; Goebbels et al., 2006; Kerrisk et al., 2013; Warren et al., 2012) which is expressed only in cortical principal neurons after embryonic day 12. For simplicity, mice expressing two copies of the floxed *erbb4* allele and Nestin-Cre (*erbb4^{flox/flox}* Nestin-Cre), will be referred to as brain specific (*bs*)-*ErbB4*^{-/-}. Mice expressing two copies of the floxed *erbb4* allele and Nex-Cre (*erbb4^{flox/flox}* Nex-Cre) will be referred to as *Nex-ErbB4*^{-/-}. No differences were observed between mice wild type for ErbB4 irrespective of Cre transgene expression and ErbB4 heterozygous mice without the Cre transgene, therefore these mice are collectively termed wild type (WT) in this study.

Dendritic spine analysis

Thy1-GFP positive animals were perfused with 25 mL of 4% PFA. Afterwards, brains were removed and postfixed in 4% PFA overnight at 4 °C. Prior to sectioning, brains were changed into 2% PFA with 30% sucrose with end over end rotation overnight at 4 °C. Brains were mounted onto a sliding freezing microtome with Tissue-tek (Sakura) cryomounting solution, sliced into 30 µm sections, and transferred into 1× phosphate buffered saline (PBS). Sections were rolled out onto glass slides and mounted in 80% glycerol. Genotypes were blinded and images of dendritic spines were captured with a Zeiss 510 Meta Confocal microscope using a 63× objective with a Z-step size of 0.7 µm during earlier studies on *bs-ErbB4*^{-/-} mice or were later captured using an UltraVIEW VoX (Perkin Elmer Life and Analytical Sciences) spinning disk confocal microscope with a Nikon 100× CFI Apo TIRF, NA 1.49, oil-immersion objective with a Z-step size of 0.2 µm for *Nex-ErbB4*^{-/-} studies. Spines were chosen primarily from the prelimbic area in the medial prefrontal cortex with some overlap in the infralimbic and cingulate areas. GFP-labeled dendritic spines were counted from branches from the apical tuft, just under the pial surface (distal), branches coming off the primary apical dendrite around layers II/III (middle), and the first or second branch near the soma (proximal). At the top of layer II/III, beneath layer I, is a band of ascending GFP positive fibers which served as a border under which middle

segments were chosen. Dendrite segments were chosen randomly from each area; however, efforts were made to avoid analyzing segments that included branches. Spines were analyzed for classification using Volocity (Perkin Elmer Life and Analytical Sciences) software. Total spine length (L), spine head width (HW), neck length (NL), and neck width (NW) were measured. Spines were classified as stubby if their length was less than 1 μm ($L < 1 \mu\text{m}$). Spines were classified as mushroom if their length was between 1 and 3 μm and their neck width was greater than two times the head width ($1 < L < 3 \mu\text{m}$, $NW > 2 \times HW$). Thin spines were between 1 and 3 μm long with a neck width that was less than two times the head width ($1 < L < 3 \mu\text{m}$, $NW < 2 \times HW$). Spines with lengths greater than 3 μm were classified as filopodia ($L > 3 \mu\text{m}$) (De Rubeis et al., 2013).

Synaptic fractionation

We used a previously described fractionation procedure (Biederer et al., 2002). Briefly, cerebral cortices and hippocampi were dissected from 3–5 mice aged P21 and homogenized in a sucrose buffer [320 mM sucrose; 10 mM HEPES (American Bioanalytical), pH 7.3] with phosphatase and protease inhibitors [Pepstatin A (10 mg/mL), Chymostatin (10 mg/mL), Leupeptin (10 mg/mL), Benzamidine (1 mM), Aprotinin (50 mg/mL), Sodium orthovanadate (1 mM), Phenylmethylsulfonylfluoride (PMSF) (1 mM), Sodium fluoride (25 mM)]. Samples were centrifuged for 10 minutes at $800 \times g$ to obtain a supernatant of crude synaptoneuroosomes, which were then centrifuged twice for 15 min at $10,000 \times g$, resuspending the pellet with fresh buffer between spins. The resulting pellet contained washed synaptoneuroosomes, which were lysed in ice cold distilled water and allowed to rotate for 15 minutes at 4°C . Samples were then centrifuged for 20 min at $24,000 \times g$ to pellet the synaptosomal membranes. The membrane pellet was then resuspended in 2 mL of 1.1 M sucrose with 10 mM HEPES. The resuspended membranes were overlaid with 5 mL of 855 mM sucrose and 2.5 mL of 320 mM sucrose into a 14 mL, thinwall, polyallomer tube to form a gradient. The step gradients were spun at $52,000 \times g$ for 2.5 hours in an SW-40 Ti swinging arm rotor, and the synaptic plasma membrane fraction at the 1.1 M and 855 mM interface was collected.

Plasmids

ErbB4 (the Cyt1 JM α isoform)-GFP was generated from the full length ErbB4 cDNA from a pLXSN-ErbB4 plasmid gifted by the Stern Lab at Yale. Briefly, the ErbB4 cDNA was amplified from the original plasmid by PCR with flanking BglII and AgeI sites using the following primers: forward, 5'- GATCAGATCT TTAATTA ACCATGCGACCGGCTACAGGA and 5'-GATCACCGGTCCCACCACAGTATT CCGGTG. After digestion with BglII and AgeI, the ErbB4 insert was ligated into pN1-EGFP.

Cell culture and transfections

Coverslips were placed into 24-well plates and coated with 20 $\mu\text{g/mL}$ poly-D-lysine (BD Biosciences) overnight, ahead of plating cells. P0-P1 mice cortices were dissected in Hank's balanced salt solution (HBSS) (Sigma) and then transferred to an enzyme solution containing 10 U/mL of papain and 0.2 mg/mL of cysteine in HBSS to incubate at 37°C . Tissue was then dissociated in Neurobasal media (Invitrogen) containing 10% fetal bovine

serum, and cells were plated at a density of ~300,000 cells/well. After several hours, the media was changed to serum-free Neurobasal media containing 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 2% B27-supplement (Invitrogen).

Cultures were transfected between 4–5 days in vitro (DIV) and cultured until 18–21 DIV. One hour before transfection, media from long-term cultures was removed, filtered, and saved. The media was replaced with 0.5 mL pre-warmed Dulbecco's modified eagle medium (DMEM) (Gibco) with no additives. 5 µg per well of pN1-EGFP (Clontech) was mixed with CaCl₂ and then added to 2× HeBS (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄·7H₂O, 15 mM D-Glucose, 42 mM HEPES) pH 7.09. 30 µL of solution was added to each well. For double labeling experiments, 9 µg/well of pN1-mCherry and 9 µg/well of pN1-ErbB4-eGFP was added to each well. After 23–25 minutes of incubation, wells were washed 3 times with DMEM and the medium was replaced with the filtered original media.

Western blot analysis

Cortices and hippocampi were dissected on ice cold glass, chopped, and then transferred to 0.5 mL of lysis buffer containing 150 mM NaCl, 5% glycerol, 1% Triton-X, 50 mM HEPES (pH 7.3), and protease and phosphatase inhibitors, as stated above. Tissue was homogenized in a dounce homogenizer, 1200 rpm for 30 passes and incubated with end over end rotation at 4 °C for 30 minutes, followed by centrifugation (20 min, 16,837 × g). Protein concentrations were determined by BCA assay (Pierce). Proteins were separated using 8% or 10% SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with anti-ErbB4 antibodies (Cell Signaling Technology, Cat# 4795, RRID:AB_2099883). Mouse anti-actin (1:10,000, EMD Millipore, Cat# MAB1501R, RRID:AB_94235) or rabbit anti-Erk1 (1:2000, Santa Cruz Biotechnology, Cat# sc-94, Lot 310, RRID:AB_2140110) were used as loading controls in standard western blotting experiments. The actin antibody (EMD-Millipore) showed only a single band around 42 kD. The Erk-1 antibody detects a double band at 44kD and 42kD representing MAP kinase p44 and to a lesser extent p42. Use of this antibody for western blotting has been documented in the antibody database (Cooper et al., 2009). In western blots of fractionated brain lysates, mouse anti-PSD95 (1:10,000, Neuromab, Cat# 75-028, RRID:AB_2292909) was used as a synaptic marker to show enrichment of the synaptic membrane. This antibody showed a prominent band of 95 kD which demonstrated enhanced intensity in fractions containing higher levels of synaptic membrane.

Immunocytochemistry

To observe ErbB4 localization, GFP negative, wild type and *bs-ErbB4*^{-/-} brains were perfused with 4% paraformaldehyde for 10 minutes, pH 7.5. Brains were allowed to post-fix for 5 hours before being changed into 30% sucrose in 1× PBS with end over end rotation overnight at 4 °C. Brains were mounted onto a sliding freezing microtome with Tissue-tek (Sakura) cryomounting solution, sliced into 25 µm sections, and transferred into 1× phosphate buffered saline (PBS). Heat-induced antigen retrieval was performed on floating sections in sodium citrate buffer (10 mM HOC(COONa)(CH₂COONa)₂, pH 6) for 20 minutes at 70° C prior to blocking with 10% goat serum and 0.2% Triton-X 100 in 1× PBS for 1 hour at room temperature. The solution was then replaced with polyclonal rabbit anti-

ErbB4 antibody (HL5721, 1:2000) (gift from the Buonanno laboratory) in blocking solution for 72 hours at 4°C. After washing 3 times, 10 minutes each, with 1× PBS, goat anti-rabbit Alexa Fluor 647 (1:500 in blocking solution) (Invitrogen) was added to each well for 3 hours at room temperature. After 3 hours, wells were washed again 3 times, 10 minutes each in 1× PBS and incubated for 24 hours with 1:200 mouse anti-CamKII, clone 6G9 (Thermo Scientific Pierce Antibodies, Cat# MA1-048, RRID:AB_325403) diluted in blocking solution. Wells were then washed 3 × 10 minutes each in 1× PBS and incubated for 3 hours with 1:500 goat anti-mouse Alexa Fluor 488 in blocking solution. After incubation, tissue was rinsed 3 times, 10 minutes each with 1× PBS. Sections were rolled out onto glass slides and mounted in 80% glycerol. Flattened z-stack images were taken with a Zeiss 710 Confocal microscope using a 63× objective with a z-step of 0.5 μm.

Antibody characterization

The polyclonal ErbB4 antibody used in immunocytochemistry to identify ErbB4 localization in cultured neurons was produced in rabbit against the intracellular domain of ErbB4. The immunogen used to create the antibody was a 204 amino acid sequence, encoded by nucleotides 1036 through 1239 of the mouse ErbB4 cDNA. This sequence is unique to ErbB4 and not shared with other EGF receptors. The specificity of this antibody was shown by immunostaining *bs-ErbB4*^{-/-} brain tissue which did not reveal specific staining for ErbB4. The immunogen used to create the CamKII monoclonal antibody was derived from rat CamKII. The antibody detects the alpha subunit of both phosphorylated and unphosphorylated CamKII, but not the beta subunit. Species reactivity was tested in mouse and use of the antibody for immunohistochemistry has been published (Ding et al., 2013; Kalita et al., 2005; Mouton-Liger et al., 2011) and cited on the antibody database.

Another monoclonal ErbB4 antibody (Cell Signaling, Cat# 4795,) was used to detect ErbB4 in western blot analyses. This antibody was produced in rabbit using a synthetic peptide corresponding to nucleotides near the carboxy terminus of the intracellular domain of the human ErbB4 receptor. Only a single 180 kD band was detected by using this antibody in immunoblots of wild type mouse cortical and hippocampal lysates. Additionally, no bands are observed when immunoblotting with this antibody from *bs-ErbB4*^{-/-} lysates (Fig. 1A) indicating no cross-reactivity with other receptors or proteins.

RESULTS

To investigate the role of ErbB4 in dendritic spines, we examined how inactivating ErbB4 in mice affected spine shape and density at different developmental stages. Since ErbB4 is required for heart development and is embryonic lethal when constitutively knocked out, we crossed mice bearing a conditional ErbB4 allele with loxP sites flanking exon 2 (Long et al., 2003) with a mouse bearing a Nestin-Cre transgene to induce recombination in neurons and glia beginning at embryonic day 11 (Isaka et al., 1999). We found no detectable ErbB4 protein in brain lysates from brain specific (*bs-ErbB4*^{-/-}) mice (Fig. 1A) showing that ErbB4 protein levels were fully reduced in the brain.

Loss of ErbB4 causes age-dependent dendritic spine loss

Wild type and *bs-ErbB4*^{-/-} (*erbb4*^{fllox/fllox} Nestin-Cre, see Materials and Methods) mice were also crossed into a transgenic *Thy1-GFP* (M1) mouse line (Feng et al., 2000) to visualize dendritic spines on cortical layer V pyramidal neurons (Fig. 1B). We imaged and analyzed dendritic spines on proximal, middle, and distal segments of secondary or tertiary apical dendrites of layer V dorsomedial prefrontal cortical neurons. This brain region is believed to correspond to the dorsolateral prefrontal cortex in humans, known to control working memory, perception, emotion, and executive functions (Moghaddam and Homayoun, 2008). At postnatal day 16 (P16), spine density was similar in WT and *bs-ErbB4*^{-/-} mice (Fig. 1C), indicating that initial spine formation proceeded normally in the absence of ErbB4 function. Overall, we found that spine density increased on all branches between P16 and P21. However, the relative increase in dendritic spine density on middle segments of layer V apical dendrites at P21 was impaired in *bs-ErbB4*^{-/-} mice, which had a 22% reduction in spine density relative to WT littermates (Fig. 1D). These middle dendrite segments reside primarily in layers II/III. Growth of new spines increased the density on branches in both genotypes between P21 and P63; however, the relative reduction in dendritic spine density in middle segments of layer V neurons persisted in *bs-ErbB4*^{-/-} mice relative to WT mice at P63 (Fig. 1E). Distal segments that extended into layer I showed no significant differences in dendritic spine density between *bs-ErbB4*^{-/-} and WT mice at any age.

ErbB4 is localized to dendritic spines in pyramidal neurons

We next examined in which cell type ErbB4 acts to control dendritic spine development. Previous reports have suggested that ErbB4 colocalizes with PSD95 in excitatory synapses (Huang et al., 2000). We measured ErbB4 protein levels in subcellular fractions made from mouse cerebral cortex and hippocampus. We found that ErbB4 was enriched 2-fold in synaptic membrane fractions (SPM in Fig. 2A) relative to both unfractionated brain lysate (UBL) and whole synaptoneurosomes fractions that include extrasynaptic membranes and cytoplasm (P2 in Fig. 2A). These data suggest that ErbB4 protein is present at the synapse.

We also directly examined ErbB4 localization in excitatory neurons. We co-transfected an ErbB4-GFP expression construct into cultured cortical neurons at 5 DIV together with mCherry to fill the neuron and analyzed localization at 18 DIV after dendritic spines had developed. We found that ErbB4-GFP was observed throughout dendrites and was also significantly enriched at dendritic spines (Fig. 2B). Quantitative analysis of fluorescence intensity using line-scans showed an enrichment of ErbB4-GFP signal relative to the co-expressed mCherry volume control at the dendritic spine (Fig. 2B). These studies reveal that ErbB4 localizes to dendritic spines in vitro, consistent with previous reports that ErbB4 regulates postsynaptic functions such as synaptic plasticity in excitatory neurons (Li et al., 2007a; Ma et al., 2003).

Recent reports have shown that endogenous ErbB4 expression is prominent in interneurons (Chen et al., 2010; Janssen et al., 2012; Neddens et al., 2011). However, our data suggest that ErbB4 can localize to spines in excitatory neurons in culture and synaptic membrane fractions from the cortex. Therefore, we examined whether endogenous ErbB4 protein could be observed in excitatory neurons. Prefrontal cortical sections were stained with an antibody

to CamKII to detect excitatory neurons together with an antibody that detects ErbB4 immunohistochemically. We detected ErbB4 in a subset of CamKII-positive neurons, even though it was present at lower levels than in adjacent interneurons. Due to the reduced staining intensity in excitatory neurons, we only detected prominent staining in the soma, most likely due to the volume of this compartment (Fig. 2C). Only faint nonspecific background ErbB4 staining was observed in sections from the negative control *bs-ErbB4*^{-/-} whole brain knockouts. These results together suggest that ErbB4 may function in excitatory neurons to affect spine density in the cortex.

Excitatory-specific deletion of *erbb4* reduces total ErbB4 in brain

ErbB4 is expressed ubiquitously in the brain and there are clear consequences of loss of ErbB4 from GABAergic interneurons (Chen et al., 2010; Fazzari et al., 2010; Okada and Corfas, 2004; Ting et al., 2011). However, our localization data strongly suggest that in addition to its important functions in interneurons, ErbB4 may act in excitatory neurons to influence dendritic spine development and stability. To test this, we used a *Nex-Cre* transgene to selectively inactivate *erbb4* in excitatory neurons of the cerebral cortex and hippocampus (*Nex-ErbB4*^{-/-} mice) (Goebbels et al., 2006; Herring et al., 2013; Kerrisk et al., 2013; Warren et al., 2012). By comparing the relative levels of ErbB4 signal in WT vs. *Nex-ErbB4*^{-/-} cortical brain lysates, we found that ErbB4 levels were reproducibly decreased 2-fold in *Nex-ErbB4*^{-/-} mice (Fig. 3). The residual ErbB4 in *Nex-ErbB4*^{-/-} mice most likely reflects ErbB4 that is present in interneurons.

Loss of ErbB4 from excitatory neurons leads to dendritic spine loss

We next examined how selective ablation of ErbB4 in excitatory neurons affected dendritic spines. In the dorsomedial prefrontal cortex, no differences in spine density were observed on apical dendrites from layer V pyramidal neurons in WT vs *Nex-ErbB4*^{-/-} at P16 (Fig. 4A). However, spine density was reduced in *Nex-ErbB4*^{-/-} mice relative to WT mice by 26% in the most proximal dendrites, corresponding to segments residing chiefly within layer V. Spine density was also decreased by 19% in middle dendrite segments of *Nex-ErbB4*^{-/-} mice at P21, which are within layers II/III. These reductions are similar to those observed in *bs-ErbB4*^{-/-} mice (compare Figs. 4C and 1D) in deep cortical layer dendrites. These reductions in *Nex-ErbB4*^{-/-} persisted at P63 in proximal dendrite branches (Fig. 4). In addition, as in the *bs-ErbB4*^{-/-} mice, there were no changes in spine density on distal segments within layer I in *Nex-ErbB4*^{-/-} mice. Since we do not detect differences in spine density earlier in development, it appears that ErbB4 functions in excitatory neurons during later stages of formation and/or stabilization of dendritic spines.

Dendritic spines are heterogeneous in shape and differences in their morphology reflect differences in function and stability. We asked whether loss of ErbB4 from excitatory neurons affected the distribution of dendritic spine shapes (e.g. mushroom, stubby, thin, filopodial), classifying spines based on total length and head width in relation to neck width (De Rubeis et al., 2013; McNair et al., 2010). Indeed, we found that mushroom-type spines were reduced by 48 ± 6% in *Nex-ErbB4*^{-/-} animals (Fig. 5B). Thus, the reduction of dendritic spine density in these mice occurs chiefly due to a loss of the more stable mushroom spines. We compared this difference to changes in the distribution of spine

classes in the *bs-ErbB4*^{-/-} brain and found that mushroom type spines are similarly reduced (Fig. 5A).

DISCUSSION

In this report, we demonstrated that the loss of ErbB4 signaling in excitatory neurons leads to reduced dendritic spine density in the prelimbic, infralimbic, and cingulate cortices within the mouse dorsomedial prefrontal cortex. These areas project to the ventral striatum and correspond to the dorsolateral prefrontal cortex, the center of executive function and working memory in humans (Groenewegen and Uylings, 2000; Moghaddam and Homayoun, 2008). While dendritic spines develop normally in wild type and *erbb4* mutant animals through P16, spine density is reduced by approximately 20% in *erbb4* mutant mice after P21. Interestingly, the extent of dendritic spine loss in both whole brain and excitatory neuron-specific *erbb4* mutant mice is of similar magnitude to that observed in schizophrenic patients in the prefrontal cortex (Black et al., 2004; Glantz and Lewis, 2000). In addition, the spine loss in *bs-ErbB4*^{-/-} mice was observed in apical dendrite segments in layer II/III (middle segments) where the majority of spines are lost in post-mortem schizophrenic brains. This comparative reduction in spine density in *bs-ErbB4*^{-/-} animals is maintained into adulthood, indicating that this initial impairment does not recover. ErbB4 protein levels persist in adult mice, albeit at lower levels than during the peak of spine maturation and pruning around 3 weeks of age. However, in *Nex-ErbB4*^{-/-} mice the early deficits in spine density in the middle segments recover by P63. By contrast, the changes in proximal dendrite branches, of *Nex-ErbB4*^{-/-} mice at P21, persist at P63. This difference in the persistence of spine loss may reflect a greater dependence of more proximal spines within deep layer dendrite segments on ErbB4 function for their formation or stability. We observed slight differences in the baseline density of spines in wild type littermate control mice from the distinct *bs-ErbB4*^{-/-} and *Nex-ErbB4*^{-/-} breeding populations, which are likely due to differences in strain background. Importantly, the reduced spine density in both *bs-ErbB4*^{-/-} and *Nex-ErbB4*^{-/-} mice is mainly due to the loss of larger mushroom-type spines, which are thought to be associated with more stable synapses (Bourne and Harris, 2007). The reduction in mushroom-type spines in *Nex-ErbB4*^{-/-} mice strongly suggests that loss of ErbB4 in excitatory neurons accounts for this effect.

While the role of ErbB4 in modulating neuronal circuitry has been studied in several animal models by disrupting either Nrg1 or ErbB4 (Fisahn et al., 2009; Garcia et al., 2000; Gerecke et al., 2004; Golub et al., 2004; Hahn et al., 2006; Huang et al., 2002; Huang et al., 2000; Krivosheya et al., 2008; Liu et al., 2007; Longart et al., 2007; Ma et al., 2003; Mei and Xiong, 2008; Neddens et al., 2009; Ozaki, 2001; Wen et al., 2010), our work is the first to directly examine a cell autonomous role for ErbB4 in the regulation of dendritic spine formation and maintenance in vivo. Our finding that loss of ErbB4 function in excitatory neurons compromises mushroom spine density is consistent with a previous report that ErbB4 knockdown in pyramidal neurons yields smaller synapses and reduced activity (Li et al., 2007a). Targeted knockdown of ErbB4 in CA1 hippocampal neurons with shRNA can attenuate NMDA and AMPA receptor mediated neurotransmission (Li et al., 2007a), suggesting that ErbB4 is present in this excitatory circuit. Furthermore, knockdown of Nrg1 from presynaptic CA3 inputs can occlude this effect (Li et al., 2007a). Similarly, ErbB2/

ErbB4-deficient hippocampal neurons exhibit defects in spine formation in culture, which is also consistent with a cell-autonomous role for ErbB4 in spine formation (Barros et al., 2009).

Neuregulin, the ligand for ErbB4 is highly expressed in the brain and appears in both pyramidal neurons and interneurons as well as in glia (Law et al., 2004; Liu et al., 2011). Strikingly, neuregulin mRNA transcripts are enriched in cortical layers III-V in the dorsal prefrontal cortex (Bernstein et al., 2006; Law et al., 2004). Several signaling pathways downstream of ErbB4 activation by neuregulin have been identified. As dendritic spines are highly enriched in the cytoskeletal protein, actin, we speculate that ErbB4 signaling can influence cytoskeletal dynamics to elicit changes in spine shape and head size. Blocking or destabilizing actin, but not microtubules, prevents neuregulin-dependent signaling and modulation of NMDA currents (Gu et al., 2005). Other recent investigations also suggest that ErbB4 signaling may activate regulators of the small GTPases such as Ras and Rac. For example, neuregulin induced increases in dendritic spine density and head size in cultured cortical neurons were found to be dependent on Kalirin-7, a Rac-GEF which activates PAK to control the actin cytoskeleton (Cahill et al., 2013). Intriguingly, Kalirin-7 is known to be downregulated in the brains of schizophrenia patients (Hill et al., 2006; Narayan et al., 2008). Finally, ErbB4 is a well-known regulator of a PI3 Kinase-AKT-GSK3 α/β kinase relay, which regulates synapse plasticity and has recently been implicated in schizophrenia models (Beaulieu, 2011; Li et al., 2007b). Gsk3 α/β can also regulate the actin cytoskeleton via phosphorylation of slingshot 2 to promote increased levels of active cofilin (Tang et al., 2011).

Although there have been reports that ErbB4 is expressed in pyramidal neurons (Bernstein et al., 2006; Gerecke et al., 2001) and can induce cell-autonomous effects in vitro (Barros et al., 2009; Cahill et al., 2013; Li et al., 2007a), it has been proposed that the reduced dendritic spine numbers observed in ErbB4-deficient mice reflect compensatory changes to neuronal circuits resulting from decreased interneuron function or number (Fazzari et al., 2010; Neddens and Buonanno, 2009; Wen et al., 2010; Yin et al., 2013). ErbB4 is prominently expressed in interneurons in developing and mature rodent and non-human primate brain (Flames et al., 2004; Neddens et al., 2011; Vullhorst et al., 2009; Yau et al., 2003). Indeed, in mice lacking ErbB4 in fast-spiking interneurons, synchrony of paired recordings between the hippocampus and medial prefrontal cortex were diminished compared to wild type (Del Pino et al.). Likewise, CA1 hippocampal neurons exhibited deficits in spine formation in mice lacking ErbB4 in parvalbumin-positive interneurons (Yin et al., 2013).

Here, we demonstrate in vivo that loss of ErbB4 from excitatory neurons leads to reduced dendritic spine density and a selective loss of mushroom-type spines. Additionally, we used immunohistochemistry to show that ErbB4 staining can be detected, albeit at low levels, in a subset of excitatory cortical neurons. The layer-specific loss of spines in the cortex due to disrupted ErbB4 expression resembles the layer-specific pathological reductions in spine density in schizophrenia, which disrupts the ability of the prefrontal cortex to properly execute plans, proper judgment, and working memory. Our findings indicate that ErbB4 function in excitatory neurons plays a critical role in dendritic spine development.

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Figure 1.

Loss of ErbB4 in the mouse brain leads to a reduction in dendritic spine density in the dorsomedial prefrontal cortex. **A.** Immunoblot of brain lysate from WT (+/+) and *bs-ErbB4*^{-/-} (-/-) mice probed for ErbB4 (top). The membrane was re-probed with anti-Erk1/2 as a loading control (bottom). The two right lanes demonstrate the complete loss of ErbB4 expression in whole brain lysates of *bs-ErbB4*^{-/-} mice. **B.** Representative images of middle segment spines from P21 wild type (+/+) and *bs-ErbB4*^{-/-} (-/-) mice. The coronal section schematic shows the areas analyzed in black. (Image credit: adapted from ©2014 Allen Mouse Brain Atlas [Internet], RRID:AB_325403). **C–E.** Quantification of spine density in wild type and *bs-ErbB4*^{-/-} animals at P16 (**C**), P21 (**D**), and P63 (**E**). n=3 animals per genotype, 15 segments (~25–35 μ m each) per animal. **p*<0.05 (student's *t*-test).

Figure 2.

ErbB4 is present in excitatory neurons and localizes to synapses and dendritic spines. **A.** Immunoblot of ErbB4 protein levels in different membrane fractions from P21 hippocampal and cortical lysates. PSD95 immunoblotting was used as a fractionation control for enrichment of synaptic membranes. UBL, unfractionated brain lysate; P2, purified synaptoneurosome fraction; SPM, synaptic plasma membrane fraction. **B.** Cultured cortical neurons transfected with ErbB4-GFP and mCherry. Neurons were co-transfected with mCherry to identify pyramidal neurons based on morphology. Image shows a dendrite shaft, labeled in magenta with ErbB4-GFP positive spines. Below, fluorescence intensity was measured using line scan analysis through selected spines to compare relative intensity in the spine shaft with the spine head. ErbB4-GFP is enriched in the spine head relative to the spine shaft. **C.** Immunostaining of wild type (WT) and *bs-ErbB4*^{-/-} (-/-) prefrontal cortical sections with anti-ErbB4 antibody (magenta) and anti-CamKII antibody (green), which detects excitatory neurons. Filled arrowheads indicate a neuron that exhibits co-labeling. Open arrowheads highlight ErbB4 positive cells with no CamKII staining, likely inhibitory neurons. Only low-level background of ErbB4 staining is observed in the ErbB4 conditional knockout mouse.

Figure 3.

ErbB4 protein levels are reduced in mice lacking ErbB4 in excitatory neurons. A. Western blot of a dilution series ranging from 100 μg to 20 μg of protein from P21 WT (top) and *Nex-ErbB4*^{-/-} (bottom) mouse cortical lysates. Membranes were probed for ErbB4 (180 kD band). B. Analysis of band intensities from WT and *Nex-ErbB4*^{-/-} immunoblots. Band intensity was measured and averaged for each protein amount loaded and genotype. n=5 immunoblots, **p*<0.05.

Figure 4.

Loss of ErbB4 from excitatory neurons leads to a reduction in dendritic spine density. A. Representative middle segment images from wild type (WT) and *Nex-ErbB4*^{-/-} (-/-) mice at different ages. B–D. Quantification of spine density at P16 (B; n=3 mice per genotype, 15 segments per mouse), at P21 (C; n=3 WT mice and 4 *Nex-ErbB4*^{-/-} mice, 15 segments per mouse. **p*<0.05, *t*-test), and at P63 (D; n=4 WT mice and 5 *Nex-ErbB4*^{-/-}, 15 segments per mouse. **p*<0.05, *t*-test).

Figure 5.

Loss of ErbB4 leads to a reduction in mushroom spines. Top panels in each figure show representative images of middle dendrite segments from layer V cortical neurons taken from (A) *bs-ErbB4*^{-/-} and WT littermate controls and (B) *Nex-ErbB4*^{-/-} and WT littermate controls at P21. Bottom panels show quantification of mushroom, thin, stubby, and filopodial spines in cortical dendrite segments from (A) WT and *bs-ErbB4*^{-/-} mice or (B) WT and *Nex-ErbB4*^{-/-} mice. **p* < 0.05. *n* = 3 mice per genotype, 3 segments per mouse.

Table 1

Primary Antibodies

Antibody Name	Immunogen	Source	Species
HER4/ErbB4 (111B2)	Human ErbB4 residues near c-terminus	Cell Signaling, 4795	Rabbit, monoclonal
PSD-95 (K28/43)	Human PSD-95 domain residues 77–299	NeuroMab, 75-028	Mouse, monoclonal
ErbB4 (HL5721)	Mouse ErbB4 residues 1036–1239 (204 aa)	Dr. Buonanno, NICHD	Rabbit, polyclonal
CamKII, alpha (6G9)	Alpha subunit of rat CamKII	Pierce, MA1-048	Mouse, monoclonal
Actin (C4)	Chicken Actin residues 50–70	Millipore, MAB1501R	Mouse, monoclonal
Erk 1 (K-23)	Rat Erk 1 subdomain XI, residues 305–327	Santa Cruz, SC-94	Rabbit, polyclonal