Impaired maturation of dendritic spines without disorganization of cortical cell layers in mice lacking NRG1/ErbB signaling in the central nervous system

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NRG1 activates receptor tyrosine kinases consisting of dimers formed by ErbB2, ErbB3, and ErbB4. Both ErbB3 and ErbB4 bind NRG1, whereas ErbB2 and ErbB4 have intrinsic tyrosine kinase activity. Functional NRG1 receptors therefore consist of ErbB4 homodimers, or of heterodimers between ErbB2, ErbB3 and ErbB4 (1). NRG1 and its receptors are expressed in the CNS and the NRG1, ErbB2, and ErbB4 genes are candidate susceptibility genes for schizophrenia (1, 2). In the central nervous system (CNS), NRG1 is thought to regulate the differentiation of radial glia and neurons, myelination, neuronal migration and synaptic function (1). However, mice with targeted deletions of NRG1, ErbB2 and ErbB4 die during embryogenesis, whereas mice lacking ErbB3 die perinatally (3–6). Thus, our knowledge of NRG1/ErbB functions in the CNS has been derived from studies using cultured cells, dominant-negative ErbB receptors, and mice partially defective in NRG1 signaling (1). No animal model lacking all NRG1 signaling specifically in the CNS has been described. We have therefore engineered ErbB2/B4-CNSko mice that lack both ErbB2 and ErbB4 (the only ErbBs with intrinsic tyrosine kinase activity) in the CNS. Surprisingly, the mutant mice show no defects in brain morphology and in the layered structure of the cerebral cortex, hippocampus, and cerebellum. Instead, the maturation of dendritic spines is affected. ErbB2/B4-deficient mice also display behavioral abnormalities that have been associated with schizophrenia-like symptoms. Clozapine treatment reverses behavioral and spine defects, indicating that perturbation of glutamatergic synapses might contribute to the behavioral abnormalities. Interestingly, reduced spine density has been observed in the brain of schizophrenia patients (7), suggesting that defects in spine maturation may constitute a risk factor for the development of schizophrenia.

Results

Normal Cortical Development and Glial-Guided Migration. To inactivate NRG1/ErbB signaling in the CNS, we crossed a mouse line homozygous for loxP-flanked (floxed) ErbB2 and ErbB4 alleles with hGFAP-CRE mice that expresses CRE in neural precursors starting at embryonic day (E) 13.5 (8). Mutant offspring will be referred to as ErbB2/B4-CNSko mice. Littermates lacking CRE or floxed alleles were indistinguishable from wild-type (WT) mice, served as controls, and will be referred to as WT. We confirmed recombination of the flox alleles and absence of ErbB2/ErbB4 proteins in the brains of mutant mice (Fig. 1 A and B). ErbB2/B4-CNSko mice were viable, had brains of normal size, and showed no defects in the layered structure of the cerebral cortex, hippocampus, and cerebellum (Fig. 1 C and D; [supporting information (SI) Fig. S1A]). Analysis with the pan-neuronal marker NeuN and the layer specific markers Cux1 (layers II–IV) and Tbr1 (layer VI) revealed no differences in neuronal density across all and within specific cortical layers (Fig. 1 E and F). These findings were unexpected, as NRG1/ErbB signaling was thought to be essential for the formation of cortical cell layers (9, 10).

Thus, to confirm our observations, we generated ErbB2/B4 double mutants using additional mice expressing CRE in neural precursors from E8.5 (Nestin-CRE) and E10.5 (Nestin-CRE, EMX1-CRE) onward (11–13). ErbB2/B4 proteins were absent in the developing CNS of the mutants (data not shown), but brain morphology and neuronal cell layers were unaffected (Fig. S1 B–D and Fig. S2 A). 5′-Bromo-2′-deoxyuridine (BrdU) pulse-labeling experiments confirmed that cell migration also progressed normally (Fig. S2 B). It has been suggested that radial glial development depends on NRG1/ErbB (10, 14, 15), yet stainings with the radial glial marker RC2 revealed no obvious defects in radial glia morphology (Fig. 1G and Fig. S3A). We also observed no changes in cell proliferation (Fig. S2C) and in the timing and levels of GFAP expression (Fig. 1H and Fig. S3 B–E), indicating that the generation of neurons and astrocytes was not affected.

Defects in Dendritic Spine Maturation. Golgi staining revealed no obvious defects in dendritic morphology in neurons of the cortex and hippocampus of ErbB2/B4-CNSko mice (Fig. 2 A and D), but dendritic spine density in pyramidal neurons in the hippocampus and cortex was reduced by 28.1% ± 3.9% and 15.8% ± 4.8%, respectively (Fig. 2 B and C; Fig. 2 E and F). To determine the mechanism causing this defect, we analyzed spine development in hippocampal cultures. After 11 days in culture, neurons from...
mutant mice had normal density of filopodia (Fig. 3A), which are believed to be spine precursors (16). Filopodial width was also unaffected, whereas their length was decreased (Fig. 3A). However, after 21 days, mature neurons from mutant mice had 54.2% ± 4.2% less spines, which were also thinner than controls but of normal length (Fig. 3B). The density of excitatory presynaptic nerve terminals, as analyzed by staining for the vesicular glutamate transporter (VGLUT) was also decreased, but VGLUT cluster size was unchanged (Fig. S4), indicating that the defects in spines were not a secondary consequence of cell death. Interestingly, spine density in ErbB2 and ErbB4 single mutants was less severely reduced (Fig. S5).

To test whether NRG1 might be limiting for spine development, we next added recombinant NRG1 (rNRG1) to WT hippocampal neurons starting on the first day of culture. After 11 days in culture, we next added recombinant NRG1 (rNRG1) to WT hippocampal neurons. After 21 days, spine density was increased by 20.6% (Fig. 3C), which are indicative of accelerated maturation toward spines (Fig. 4A). After 21 days, spine density was increased by 20.6% ± 4.5% (Fig. 4B) and paralleled by an increase in VGLUT cluster staining (Fig. 4C).

When rNRG1 was added only at days 18 and 20, after dendritic ErbB2 terminals in hippocampal neurons, maturation of dendritic spines and excitatory presynaptic nerve terminals was similarly increased (Fig. S6). Our results show that ErbB2/B4-mediated Nrg1 signaling facilitates the maturation of dendritic spines and excitatory presynaptic nerve terminals in hippocampal neurons.

**Loss of NRG1/ErbB Signaling Disrupts Association of NMDA Receptors with PSD-95.** ErbB2 and ErbB4 associate with PSD-95, which also binds and clusters NMDA receptors (NMDAr) to promote spine maturation (17–20). Thus, we hypothesized that NRG1/ErbB signaling may modulate PSD-95/NMDAr interactions to promote the formation of mature dendritic spines. Consistent with this model, the number of PSD-95 and NMDAr NR1 clusters was reduced in mutant hippocampal neurons (Fig. 5A and B). Cluster size was unchanged (Fig. S7A), but co-localization between NR1 and PSD-95 clusters was affected (Fig. 5A and C). Co-immunoprecipitation experiments confirmed that complex formation between NMDAr and PSD-95 was decreased (Fig. 5D), even though NMDAr and PSD-95 levels were unchanged in the mutants (Fig. S7B and C). We conclude that interactions of NMDAr with PSD-95 are perturbed in the absence of NRG1/ErbB signaling, which is predicted to cause spine loss.

**Clozapine Reverses Behavioral and Spine Defects.** Defects in synaptic density are expected to affect the function of neuronal circuits. We therefore analyzed the performance of ErbB2/B4-CNSko mice in several behavioral paradigms, including those that have been reported to function as read-outs for schizophrenia-like symptoms. In the open-field task, ErbB2/B4-CNSko mice showed normal locomotor behavior (Fig. 6A) but stayed longer in the central zone of the open field (Fig. 6B), indicative of decreased anxiety. Next, we tested the mice in the resident-intruder assay. Mutants engaged for longer times in aggressive activities (Fig. 6C). Finally, the prepulse inhibition (PPI) test, a psychometric measure for sensory gating, showed that mutant males had lower PPI levels than WT males (Fig. 6D). No difference was observed among females (data not shown). Interestingly, increased aggression and lower PPI levels are abnormals observed in some schizophrenia patients (21–23) and gender differences in PPI have been reported in both healthy and...
schizophrenic humans (24). Moreover, clozapine administration, which is used to treat schizophrenia in humans (23), alleviated behavioral responses in ErbB2/B4-CNSko mice (Fig. 6 C and D), and restored dendritic spine numbers and width in mutant neurons (Fig. 6E).

Discussion

Our findings challenge the current view of the role of NRG1/ErbB signaling in the CNS. Studies with cells in culture, organ explants, and mice expressing dominant negative ErbB receptors suggest that NRG1 might promote the generation of radial glia and the migration of neurons along glial fibers (9, 10, 14, 15). However, these processes were unaffected in ErbB2/B4-CNSko mice, where all endogenous ErbB-mediated NRG1 signaling was abolished specifically within the CNS. ErbB2/B4 were effectively inactivated from early stages of cortical development, and similar results were obtained with four CRE lines. One of the lines, Nestin8-CRE, expresses CRE already at E8.5 in the neuroepithelium, before formation of radial glial cells (11–13). One explanation for the difference from earlier findings is that NRG1 may have redundant functions with other signaling pathways. Overexpression of dominant negative ErbB receptors that were used in previous studies may have affected these pathways as well.

Instead, we demonstrate that NRG1/ErbB signaling regulates dendritic spine maturation. ErbB4 binds to PSD-95 and ErbB2 associates with PSD-95 via Erbin (19, 20). PSD-95 in turn binds to NMDAr and controls the incorporation of glutamate receptors into synapses, promoting spine formation (17, 18). In cultured hippocampal neurons, NRG1/ErbB4 can activate activity-dependent plasticity of glutamatergic synapses (25). We now show that NRG1/ErbB signaling is critical for the consolidation of dendritic filopodia.
Fig. 4. Exogenous nRG1 accelerates dendritic spine maturation. (A) WT hippocampal neurons at DIV11 transfected with eGFP (green) were treated with nRG1 or vehicle (veh). In the presence of nRG1, the density and width of filopodia-like protrusions (arrows) increased, but not their length: density [protusions/10 μm]: WT + nRG1 1.36 ± 0.04, WT + veh 1.39 ± 0.06, P > 0.05; n = 768 protrusion from eight WT + nRG1 neurons, n = 536 protrusions from 8 WT + veh neurons). (B) WT hippocampal neurons at DIV21 treated with nRG1 or vehicle. The density and width of spines (arrowheads) was increased by 861 clusters from 9 WT neurons; WT + nRG1 4.19 ± 0.01, #protu./10 μm: WT + nRG1 1.33 ± 0.01, WT + veh 0.28 ± 0.01, ***P < 0.001; length [μm]: WT + nRG1 0.33 ± 0.01, WT + veh 0.35 ± 0.01, **P < 0.01; width [μm]: WT + nRG1 0.44 ± 0.01, WT + veh 0.39 ± 0.01, ***P < 0.001; length, μm]; WT + nRG1 0.92 ± 0.01, WT + veh 0.88 ± 0.01, P > 0.05; n = 1142 spines from 9 WT + nRG1 neurons, n = 861 spines from nine WT + veh neurons). (C) Immunostaining for VGLUT (blue) in DIV21 WT hippocampal neurons treated with nRG1 or vehicle. Blue channel is shown separately in gray. In the presence of nRG1, VGLUT cluster density and size was increased: density [clusters/10 μm]: WT + nRG1 8.31 ± 0.34, n = 851 clusters from 18 neurons; WT + veh 7.05 ± 0.35, n = 582 clusters from 13 neurons, *P < 0.05; size, pixels: WT + nRG1 71.46 ± 3.98, WT + veh 52.68 ± 2.76, ***P < 0.001). Scale bars, 10 μm.

Fig. 5. Loss of ErbB2/B4 affects interactions between NMDAr and PSD-95. (A) Hippocampal neurons from WT and ErbB2/B4-CNSko mice transfected with eGFP (green) were stained at DIV21 with antibodies to PSD-95 (red) and NMDAr subunit NR1 (blue). Higher magnifications with red and blue channels are also shown separately in gray. (B) The density of PSD-95 and NR1 clusters was reduced in mutants. NR1 density (clusters/10 μm): WT 6.92 ± 0.45, n = 465 clusters from 10 neurons; ErbB2/B4-CNSko 4.17 ± 0.43, n = 353 clusters from 11 neurons; ***P < 0.001; PSD-95 density (clusters/10 μm): WT 6.9 ± 0.52, n = 464 clusters from 10 neurons; ErbB2/B4-CNSko 4.19 ± 0.36, n = 347 clusters from 11 neurons, ***P < 0.001. (C) In ErbB2/B4-CNSko neurons the percentage of co-localization of NR1 and PSD-95 clusters was reduced (%): WT 23.25 ± 0.85, ErbB2/B4-CNSko 16.88 ± 1.17, ***P < 0.001). (D) Co-immunoprecipitation of NR1, NR2A or NR2B with PSD-95 revealed a 64.8 ± 8.1% (*P < 0.05), 34.5 ± 2.5% (**P < 0.01), and 30.2 ± 5.7% (***P < 0.01) decrease of protein interactions in mutants. Reverse co-immunoprecipitation of PSD-95 with NR1 also showed a reduction of 18.2 ± 1.7% (**P < 0.01). For each co-immunoprecipitation assay, the amount of immunoprecipitated protein revealed by immunoblotting is also presented. Negative controls for co-immunoprecipitation assays are shown in Fig. 5C. Scale bars, 10 μm.
Fig. 6. Behavioral abnormalities in ErbB2/B4-CNSko mice and spine loss are attenuated by clozapine treatment. (A, B) In open-field test, ErbB2/B4-CNSko mice showed no difference in distance traveled compared with WT (A; WT 2418 ± 500 cm, n = 17 animals; ErbB2/B4-CNSko 2218 ± 374 cm, n = 15 animals; P > 0.05), but remained longer in the central zone of the open field (B; WT 17 ± 2% of time, ErbB2/B4-CNSko 27% ± 4% of time, *P < 0.05). (C) Resident intruder assay showed that ErbB2/B4-CNSko mice engaged for longer time in aggressive behavior (biting, kicking, wrestling) compared with WT (WT 29.5 ± 9.3 seconds, n = 7 animals; ErbB2/B4-CNSko 122.8 ± 41.6 seconds, n = 5 animals; *P < 0.05). Clozapine (cloz) treatment decreased aggression in ErbB2/B4-CNSko mice to WT levels (ErbB2/B4-CNSko + cloz: 6.37 ± 5.99 seconds; WT without clozapine: 29.5 ± 9.3 seconds, P > 0.05), without affecting basal aggression in WT (WT + clozapine: 43.3 ± 11.2 seconds; WT without clozapine: 29.5 ± 9.3 seconds, P > 0.05). (D) In a PPI test, ErbB2/B4-CNSko mice showed reduced PPI compared with WT (WT 78.6% ± 4.3% PPI, n = 7 animals; ErbB2/B4-CNSko 57.4% ± 8.4% PPI, n = 5 animals; *P < 0.05). This difference was abolished after clozapine treatment (WT 80.7% ± 11.6% PPI, ErbB2/B4-CNSko 77.7% ± 6.2% PPI; P > 0.05). (E) Hippocampal neurons from WT and ErbB2/B4-CNSko mice at DIV1 treated with clozapine or vehicle (veh). In neurons from WT mice, clozapine treatment resulted in an increase in spine density, but spine width and length were unaffected: (density, spines/100 μm: WT + veh 10.56 ± 0.46, WT + cloz 12.7 ± 0.37, ***P < 0.001; (width, μm): WT + veh 0.51 ± 0.01, WT + cloz 0.51 ± 0.01, P < 0.05; (length, μm): WT + veh 1.13 ± 0.03, WT + cloz 1.08 ± 0.02, P > 0.05; n = 641 spines from 11 WT + veh neurons, n = 1058 spines from 13 WT + cloz neurons). In neurons from ErbB2/B4-CNSko mice, clozapine increased spine density, width, and length: density, spines/100 μm: ErbB2/B4-CNSko + veh 5.88 ± 0.35, ErbB2/B4-CNSko + cloz 8.83 ± 0.29, **P < 0.001 (width, μm): ErbB2/B4-CNSko + veh 0.42 ± 0.01, ErbB2/B4-CNSko + cloz 0.5 ± 0.01, ***P < 0.001 (length, μm): ErbB2/B4-CNSko + veh 1.06 ± 0.03, ErbB2/B4-CNSko + cloz 1.2 ± 0.03, **P < 0.01; n = 518 spines from 13 ErbB2/B4-CNSko + veh neurons, n = 678 spines from 11 ErbB2/B4-CNSko + cloz neurons. Scale bars, 10 μm.

**Generation and Analysis of Mice.** ErbB2/B4-CNSko mice were generated by crossing mice homozygous for floxed (fl) ErbB2 and ErbB4 alleles (27, 28) with different CRE lines as indicated. Histology and biochemistry were carried out as described (12, 29). The source of all antibodies used is listed in the supplement. Behavioral analysis was carried out with 1.5–7-month-old mice following puberty (12, 29). The source of all antibodies used is listed in the supplement. Behavioral analysis was carried out with 1.5–7-month-old mice following puberty (12, 29). The source of all antibodies used is listed in the supplement.

**Hippocampal Cultures and Imaging.** Hippocampal neurons from P1 mice were cultured, transfected, and stained as described (32). Ten-μM human NRG1-β1 EGF domain (R&D Systems) or one μM clozapine (Sigma) was added when indicated.