Development/Plasticity/Repair

Neuregulin Signaling Is Dispensable for NMDA- and $GABA_A$-Receptor Expression in the Cerebellum *In Vivo*

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Neuregulin-1s (NRG-1s) are a family of growth and differentiation factors with multiple roles in the development and function in different organs including the nervous system. Among the proposed functions of NRG-1s in the nervous system is the regulation of genes encoding certain neurotransmitter receptors during synapse formation as well as of other aspects of synaptic function. Here, we have examined, in granule cells of the cerebellum *in vivo*, the role of NRGs in the induction of NMDA receptor (NMDA-R) and $GABA_A$ receptor ($GABA_A$-R), which are thought to be induced by NRG-1 secreted by the synaptic inputs. To this end, we used the Cre/loxP system to genetically ablate the NRG receptors ErbB2 and ErbB4 selectively in these cells, thus eliminating all NRG-mediated signaling to them. Unlike previous reports using cultured granule cells to address the same question, we found that the developmental expression patterns of the mRNAs encoding the NR2C subunit of the NMDA-R and the β2-subunit of the $GABA_A$-R is normal in mice lacking the NRG receptors ErbB2 and ErbB4. Likewise, no alterations in cerebellar morphology nor in certain aspects of cerebellar wiring were resolved in these mutants. We conclude that NRG/ErbB signaling to the granule cells is dispensable for the normal development of their synaptic inputs.

Key words: **synapse formation; development; neuregulin; cerebellum; granule cell; NMDA receptor**

Introduction

Signal transmission at chemical synapses depends on the matching of the neurotransmitter released from the presynaptic nerve terminal with a type of postsynaptic receptor recognizing the transmitter. For a neuron receiving diverse types of synaptic inputs this implies that the formation of a synapse requires, in response to molecular interactions with respective presynaptic neurites, the expression of specific genes from its nucleus and the delivery of their products to the respective synaptic contacts (Craig et al., 2006).

Most attempts to identify the presynaptic signaling molecules inducing the postsynaptic expression of such genes have been based on assessing changes in the levels of neurotransmitter receptor gene expression in cultured neurons in response to applying the candidate factor. At the best investigated synapse, the neuromuscular junction (NMJ), such experiments have strongly suggested that neuregulins (NRGs) from the motor neuron, by activating ErbB receptors in the muscle surface, mediate the neural control of synapse-specific expression of ACh receptors (AChRs) in the muscle (for review, see Falls, 2003). However, genetic ablation of neuro-muscular NRG/ErbB signaling has subsequently shown that NRGs are dispensable for NMJ formation and maintenance *in vivo* (Escher et al., 2005).

Based on their ability to elevate neurotransmitter receptor expression levels in cultured brain slices, neuregulin-1 isoforms have also been implicated in synapse-specific receptor expression in the central and peripheral nervous systems (for review, see Mei and Xiong, 2008). Specifically, certain NRG-1 isoforms are thought to induce AChRs in sympathetic ganglion cells (Yang et al., 1998) and the switch in NMDA receptor (NMDA-R) subunit expression from NR2B to NR2C (Ozaki et al., 1997) observed in postnatal cerebellar granule cells (Feldmeyer and Cull-Candy, 1996; Cull-Candy and Leszkiewicz, 2004; Karavanova and Buonanno, 2007). In support of this idea, granule cells maturing in the internal granule layer express NRG receptors (Rieff et al., 1999), and mossy fibers (MFs) and Golgi cells innervating them (Corfas et al., 1995) express NRG1. However, when applied to dissociated granule cells in culture, exogenous NRG-1 was without effect on NR2C expression. Instead, it induced expression of the β_2-subunit of $GABA_A$ receptor ($GABA_A$-R), and it promoted outgrowth of their neurites in culture and *in vivo* (Rieff et al., 1999; Rieff and Corfas, 2006).

In addition to the suspected role of NRGs in transmitter receptor expression their effects include an influence on key developmental processes such as the proliferation, migration and fate determination of neuronal and glial precursors (Falls, 2003). The conflicting findings on NR2C expression reported above could thus be explained by differences in culture conditions which in turn may affect the mode of receptor expression indirectly. For example, depending on the K^+ concentration in the external medium of granule cells developing in culture, a range of gene expression phenotypes can be produced (Vallano et al. 1996; Mellor et al., 1998).

Here, we have examined the roles of NRG/ErbB signaling in NMDA-R and $GABA_A$-R expression in cerebellar granule cells

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during development *in vivo* by genetic ablation of ErbB receptors selectively in these cells. Analysis of mutant cerebella showed no difference in the expression levels of mRNAs encoding NR2C and $GABA_A$-R $\beta 2$ subunits during postnatal development, and the cerebellar morphology appeared normal. Likewise, synaptic wiring of climbing fibers (CFs) to Purkinje cells, a process thought to be affected by granule cell input to the Purkinje cells (Hashimoto and Kano, 2005), appeared undisturbed. Experiments in organotypic slice cultures confirmed that NR2C induction during postnatal development in granule cells can occur independently of NRG/ErbB signaling. These results strongly suggest that NRG/ErbB signaling at nascent synaptic inputs to cerebellar granule cells is dispensable for normal developmental $GABA_A$-R and NMDA-R expression in these cells *in vivo*.

Materials and Methods

Animals. Selective inactivation of *erbb2* and *erbb4* genes in cerebellar granule cells was achieved by crossing the mouse line *BACα6Cre-C*, which expresses Cre recombinase with high selectivity in cerebellar granule cells under the control of the *$GABA_A$-R α6* subunit gene promoter (Aller et al., 2003), with the *erbb2fl/fl;erbb4fl/fl* line described by Escher et al. (2005). In tissue sections Cre-mediated recombination was examined by crossing the *BACα6Cre-C* line with a lacZ reporter line (Tsien et al., 1996).

Genotyping and Southern blot analysis. Mice were bred and experiments were conducted according to the Swiss animal protection laws. Extraction of genomic DNA and genotyping were performed as previously described (Aller et al., 2003; Escher et al., 2005). Genomic DNA for Southern blots were extracted from muscle as for genotyping (above) but was subjected to a phenol/chloroform extraction before precipitation with isopropanol. Ten micrometers of restriction enzyme digested (overnight) genomic DNA was loaded in each slot of a 0.8% TBE (Tris-borate/EDTA) gel and run for 3 h at 80 mA. Southern transfer onto Hybond N (Amersham) was done according to standard procedures in 10× sodium chloride-sodium citrate buffer (SSC) and UV cross-linked in a stratalinker 2400 (Stratagene). Radioactive probes were prepared by labeling a gel-purified PCR fragment using the megaprime kit (Amersham) and [^{32}P]dATP. Free nucleotides were removed using mini Quick spin columns (Hofmann-La Roche). Primer sequences for PCR fragment of the neo probe (*erbb4* allele) were 5′-TCAGAAGA ACTCGTCAAGAAGG-3′ and 5′-ATGATTGAACAAGATGGATTGC-3′.

Prehybridization for 1 h and hybridization overnight with 10^6 cpm denatured probe/ml was performed at 65°C in rapid-hyb buffer (Amersham). After final washes at 65°C in 0.1× SSC, 0.1% SDS, membranes were exposed for 3 h and scanned in a phosphoimager. Densitometric estimations of the fraction of recombined bands were done using the phosphoimager software subtracting the respective background for each lane.

Cerebellar slice cultures. Cultures were prepared from B6CF1 mice as described previously (Kapfhammer, 2004; Sirzen-Zelenskaya et al., 2006). Animal experiments were be performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and permitted by Swiss authorities. Briefly, mice were decapitated at postnatal day 7 (P7) or P8, their brains were aseptically removed and placed into ice-cold preparation medium [modified Eagle's medium (MEM), 2 mM glutamax, pH 7.3]. The cerebellum was dissected and the meninges were removed. Sagittal sections (350 μm) were cut using a McIllwain tissue chopper under aseptic conditions. Slices were separated, transferred onto permeable membranes (Millicell-CM, Millipore) and incubated on a layer of serum containing culture medium (25% MEM, 25% horse serum, 2 mM glutamax I, 3.6 mM glucose) in a humidified atmosphere with 5% CO_2 at 37°C. For some culture experiments a serum-free medium was used consisting of Neurobasal A medium containing B27 supplement (Invitrogen) and 2 mM glutamax I (Adcock et al. 2004). The medium was changed every 2–3 d for a total of 7–14 d. The following pharmacological treatments were used: Neuregulinβ1 (R&D Systems) at 5 nM (tested for biological activity in cultured myotubes) (Escher et al., 2005), Tetrodotoxin (Sigma) at 2 μM, dl-2-amino-5-phosphonovaleric acid (APV, Tocris) at 100 μM, the ErbB4 and ErbB2 blockers AG1478 and AG879, respectively (Calbiochem) at 5 μM. Pharmacological agents were added at each medium change, starting 2–4 d after setting up the culture. At the end of the culture period slices were harvested from the membrane using a cell scraper and immediately frozen in liquid nitrogen for later analysis by RT-PCR.

Immunohistochemistry. For analysis of the cerebellar morphology 4 months old control and *erbb2/erbb4*-deficient mice were killed by perfusion with 4% paraformaldehyde and the cerebellum was sectioned on a cryostat at 20 μm. Sections were incubated free floating with the following antibodies: rabbit anti-calbindin D28K (Swant, 1:1000), mouse monoclonal anti-NeuN (Millipore, 1:500), rabbit anti-GFAP (Dako, 1:1000), and rabbit anti-vGlut2 (Synaptic Systems, 1:1000). The staining was visualized with anti-mouse and anti-rabbit AlexaFluor 488 and 568 second antibodies (Invitrogen).

RNA isolation and quantitative PCR. Mice were killed at different developmental stages through CO_2 exposure. RNA from tissue samples (cerebellar slice cultures and cerebellum) were isolated with TRIzol (Invitrogen) using the FastRNA tubes green (Q-Biogene) according to their protocol. DNase I (Promega) treatment and reverse transcription was performed on 1–5 mg total RNA with oligo dT primers and superscript reverse transcriptase from Invitrogen according to their protocol. For a given set of experiments, the same amount of total RNA from each sample was used for cDNA synthesis. Quantitative PCR was performed with SyBR Green mix (Applied Biosystems) using the applied biosystems machine with a two step PCR (60°C, 1 min and 95°C 15 s) for 40 cycles. The quantitative PCR mix was prepared as follows: 15 μl SyBR Green mix, 0.15 μl of a 100 pm solution each of forward and reverse primer, 1 μl of cDNA synthesized according to the reverse transcription kit protocol from Invitrogen. Each sample for real time PCR was done in triplicate and the mean of the resulting three values were taken. The following primers were used for NR2C, $GABA_A$-R $\beta 2$, and rL8 (ribosomal protein L8) amplifications: NR2C forward 5′-CCTGGTCTACTGGA-AACTTC-3′ and reverse 5′-AGCATCTTCAGCACATTGGC-3′; $GABA_A$-R $\beta 2$ forward 5′-TTTTCCTTCTTCAACATCGTCTAT-3′ and reverse ′-GTACAACTGTTTGAAGAGGAATCC-3′; and rL8 forward 5′-ACTGGACAGTTCGTGTACTG-3′ and reverse 5′-GCTTCACT-CGAGTCTTCTTG-3′.

X-Gal staining. Cerebellar cryosections (10 μm) of *BACα6Cre-C* × *R26R* (Rosa 26 Cre reporter) mice were fixed in 4% paraformaldehyde (PFA)/PBS and washed in 2 mM $MgCl_2$/PBS, pH 7.3 for 1 h. They were then incubated with the X-Gal staining solution (5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2 mM $MgCl_2$, 1 mg/ml X-Gal (Promega) overnight at 37°C. Samples were washed 3 × 5 min in PBS, dehydrated in serial alcohols, and analyzed.

Cre/DAPI staining. Cerebellar cryosections (6 μm) of *BACα6Cre-C* mice were fixed in 4% PFA/PBS, blocked in 10% normal goat serum/PBST for 1 h and incubated overnight with anti-Cre antibody [1:3000, gift from E. Casanova, Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Vienna, Austria] at 4°C. After washing 3 × 15 min with PBST, sections were treated with Cy2-conjugated goat anti-rabbit IgG (Invitrogen, 1:500) for 1 h at room temperature and with DAPI (Sigma, 1:10,000) for 5 min. They were finally rinsed in PBS and coverslipped with Citifluo.

Western blotting. Cerebella of adult wild-type (wt) and *BACa6Cre-C; erbb2fl/fl;erbb4fl/fl* mice were homogenized in lysis buffer containing 0.32 M sucrose, 4 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, protease inhibitor mixture (Roche). One-third of the protein lysate were loaded in each lane of a 6–15% gradient polyacrylamide gel and run under constant voltage (100 V) before being transferred onto a PVDF membrane. The membrane was blocked in 1× PBS containing 5% milk powder and incubated for 2 h in 1×PBS containing 2.5% milk powder with the following primary antibodies: Neu (3B5; sc33684, Santa Cruz Biotechnology), 1:300), ErbB4 (s.c.-283, Santa Cruz, 1:300). Goat anti-rabbit (for ErbB4) and anti-mouse (for ErbB2) IgG HRP-conjugated (Santa Cruz, 1:3000) were used as secondary antibody and β-tubulin (PharMingen, BD Biosciences, 1:2000 for ErbB4 blot and 1:6000 for ErbB2 blot to reduce signal intensity of β-tubulin) as loading control. Longer exposures were needed to detect ErbB2. It was important to use fresh cerebellar lysate for ErbB4 detection in Western blots.

Electrophysiology. Mice of either sex older than P21 were used to study the CF innervation of Purkinje cells. CF EPSPs and the dendritic transient of intracellular free calcium ($\Delta[Ca^{2+}]_i$) associated with a CF–EPSP were measured using patch-clamp recordings as described by Canepari et al. (2008). Briefly, $\Delta[Ca^{2+}]_i$ signals in cells loaded with 1 mM Fura-FF were derived from relative changes of fluorescence excited at 387 ± 6 nm wavelength and detected with a NeuroCCD-SM fast charge-coupled device camera (RedShirtImaging). The stimulation of CF–EPSPs was achieved by delivering short current pulses with patch pipettes filled with extracellular solution and positioned in the granule cell layer in the proximity of the recording Purkinje neuron. In additional experiments, CF EPSCs were recorded: only inputs with marked paired pulse depression were used for the analysis. The confidence interval for multiclimbing fiber innervation was obtained using the modified Wald method (Agresti and Coull, 1998).

Two wild-type and three knock-out mice at P16 were used to study morphology and NMDA-mediated currents in cerebellar granule cells. To count dendrites, neurons were filled with 500 μM Bis-Fura-2 and reconstructed by two-photon microscopy using a tunable, mode-locked titan sapphire laser (MaiTai HP, Spectra Physics) and a confocal laser-scanning system (FV300, Olympus). MF EPSCs were elicited in Mg^{2+}-free solution containing 10 μM bicuculline and recorded at −80 mV voltage clamp (after correction for junction potential). AMPA- and NMDA-mediated components of the MF–EPSC were blocked with 10 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide (NBQX) and 50 μM D-AP5 [D-(−)-2-amino-5-phosphonopentanoic acid], respectively. The time course of the NMDA-mediated component was fitted with a biexponential function. Traces used for analysis and used for illustration were the averages of 16–32 trials.

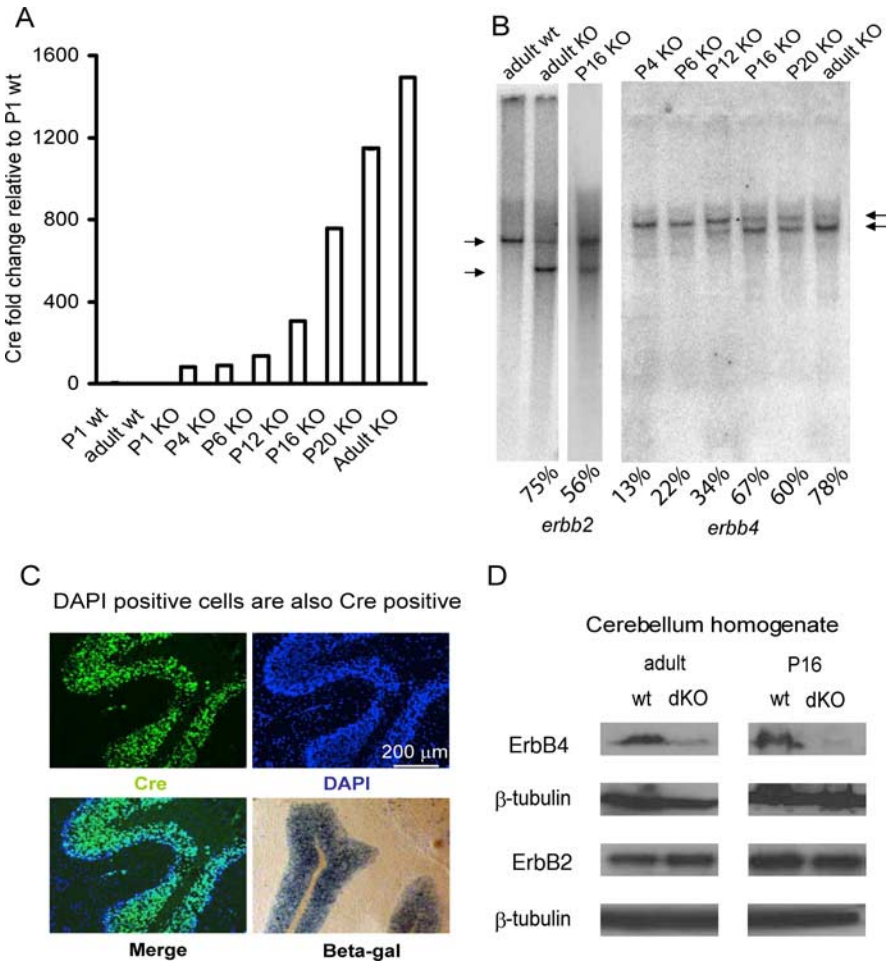

Figure 1. Expression of Cre recombinase and inactivation of *erbb2/erbb4* genes in *BACa6Cre-C;erbb2fl/fl;erbb4fl/fl* mice. ***A***, Increasing levels of Cre mRNA expression in cerebellum during postnatal development with a maximum level in the adult. Quantitative analysis by qRT-PCR. ***B***, Southern blot demonstrating Cre-mediated recombination of the *erbb2* and *erbb4* floxed allele with a maximum amount of recombination in the adult. ***C***, Expression of Cre recombinase and Cre-driven recombination are largely confined to granule cells as revealed in parasagittal section from adult mutant cerebellum. Comparison of Cre-expressing cells and of DAPI stain reveals Cre expression in >95% of cells in internal granule layer. Most of the DAPI-stained Cre-negative nuclei at the outer margin of the granular layer are likely to represent somata of Purkinje cells, of Golgi cells, and of Bergmann glial cells, which are all located at the interface of the granular layer and the Purkinje cell layer. ***D***, Western blots from adult cerebella of *erbb2/erbb4*-deficient mice show substantial abolishment of ErbB4, but little change in ErbB2 protein (see Materials and Methods). Right panel, ErbB4, but not ErbB2 in mutant cerebellum, is reduced at P16.

Results

Conditional deletion of *erbb2* and *erbb4* receptor gene subunits begins at P5

NMDARs are composed of an obligatory NR1 subunit in combination with at least one type of NR2 subunit (NR2A-D) which confer distinct activation, gating and ion conduction properties to the functional NMDAR channel (Feldmeyer and Cull-Candy, 1996; Cull-Candy and Leszkiewicz, 2004). *In situ* hybridization suggests that their subunit composition changes during development (Monyer et al., 1994). In general, NR2B and NR2D subunit mRNAs are expressed strongly during embryonic and early postnatal life, and decrease during maturation. In contrast, the levels of NR2A and NR2C mRNAs are low at early postnatal stages and increase after the first postnatal week. Inserting a lacZ reporter at the NR2C translation initiation site, under the control of the endogenous NR2C promoter, showed that beginning at P5–P6 of cerebellar development, NR2C is expressed in a caudo-rostral sequence in granule cells of the internal granular layer, to reach a final value in the entire layer around P30 (Karavanova et al., 2007). To see whether NR2C expression is regulated by NRGs, we inactivated its receptors, ErbB2 and ErbB4, in granule cells by crossing mice doubly homozygous for loxP-flanked exons of *erbb2* and *erbb4* alleles with a mouse line that begins to express Cre recombinase under the control of the $GABA_A$-R α6 subunit gene promoter in postmigratory granule cells of the emerging internal granule layer at P5–P6, i.e., at a stage when the NR2C and α6 genes are first expressed (Mellor et al., 1998; Laurie et al., 1992b; Karavanova et al., 2007). The Cre mouse line used (*BACα6Cre-C*) has been described by Aller et al. (2003).

In a first series of experiments, we examined the penetration of the recombination to be expected during postnatal cerebellar development. Cre mRNA expression levels were measured by qRT-PCR and Cre-mediated inactivation of ErbB4 receptors by Southern blotting. Cerebella were excised at various stages from P1 to adulthood. As expected, no signal was observed in P1 and adult wt controls. In contrast, a low level of Cre was observed at P1–P4 that subsequently increased strongly into adulthood (Fig. 1*A*). In accordance, excision of the loxP-flanked sequence of *erbb4* began at P4 and was nearly complete in the adult (Fig. 1*B*). Analysis of the bands by phosphoimaging in Figure 1*B* suggests

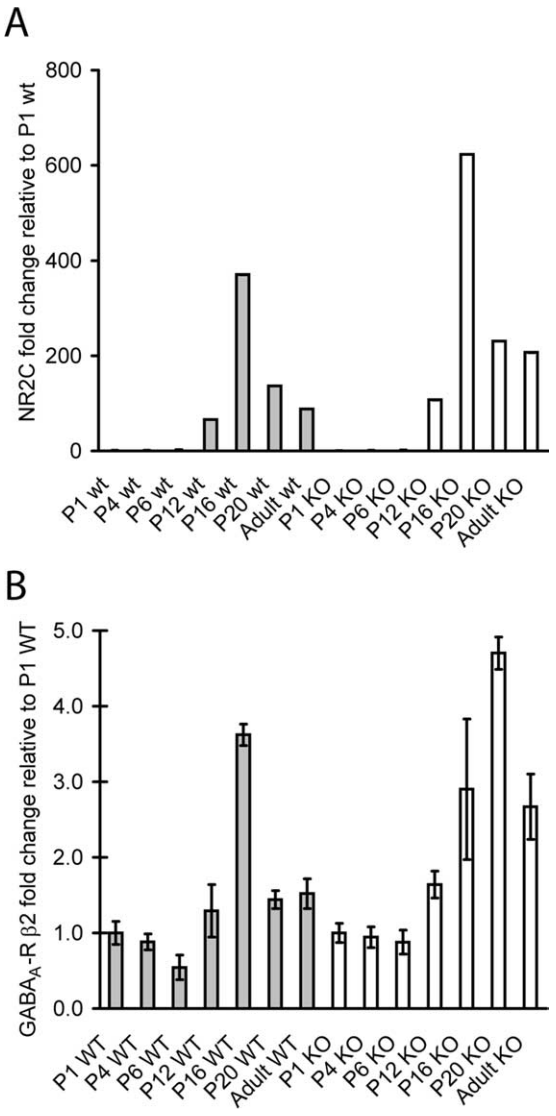

Figure 2. ***A***, Expression of NR2C mRNA in postnatal development in both wt and *erbb2/erbb4* dKO mice are similar. All values are normalized to ribosomal protein L8 (rL8) expression and expressed as fold change relative to P1 wt. All values are from triplicate measurements. Similar results were found in three independent experiments. ***B***, Same for GABA$_A$-R β2 subunit mRNA.

that in the adult, almost 80% recombination is achieved. A similar degree of recombination was observed for the *erbb2* alleles (Fig. 1*B*). This is similar to the reported percentage of granule cells out of the total number of cells in the cerebellum (~90%) (Korbo et al., 1993), again indicating recombination of *erbb2/erbb4* in most granule cells.

Next, we examined the penetration of Cre transgene expression and of Cre-mediated recombination histologically in adult mutant cerebella. Sagittal sections were stained with an antibody for Cre, and the number of Cre-positive cells in the granular layer was compared with the number of nuclei stained by DAPI. Such comparison showed that >95% of cells in the granular layer expressed Cre (Fig. 1*C*). Crossing the Cre line with a reporter line expressing β-Gal in response to Cre-mediated recombination of a translational stop sequence suggested close to full penetration of recombination in the granular layer (Fig. 1*C*). This finding was confirmed by Western blotting: ErbB4 protein was substantially reduced in *erbb2/erbb4* mutant cerebella, whereas no difference was observed with ErbB2 (Fig. 1*D*). This is in accordance with the respective levels of ErbB2 and ErbB4 expression predicted from *in situ* hybridization experiments (Ozaki et al., 1998) and the large percentage of granule cells. Finally, a significant difference in ErbB4, but not in ErbB2 protein levels, was observed at P16 (Fig. 1*D*) when recombination is ~60%. This indicates that ErbB4 on the cell surfaces is degraded rapidly after DNA recombination and, given that granule cells (GCs) express no ErbB3 (Ozaki et al., 1997), that NRG/ErbB signaling to the GCs declines rapidly upon Cre-dependent recombination.

Given that *erbb2/erbb4* alleles are ~60% recombined at P16 and that few synapses are formed by mossy fibers before the third postnatal week in the rat (Altman, 1972), our findings combined indicate that ErbB2/ErbB4 ablation begins before or at the time when synapses form even considering that development proceeds a few days faster in mouse compared with rat. The extent of ablation in our mouse model is thus sufficient to resolve impairment of NR2C expression in cerebellar granule cells, if it is dependent on NRG/ErbB signaling *in vivo*.

The temporal NR2C expression in the cerebellum is indistinguishable from normal in the absence of ErbB2/ErbB4 signaling *in vivo*

Expression of NR2C mRNA was assayed by qRT-PCR at different stages of postnatal development, relative to that at P1. In both wt and in *erbb2/erbb4* mutant mice, similar developmental expression patterns of NR2C mRNA were seen: NR2C mRNA levels were increased measurably at P12, reached a maximum at P16 and subsequently declined (Fig. 2*A*). Similar patterns were observed in three developmental series of cerebella, with levels in mutants slightly exceeding those in wt in two of the three experiments. Given that this variability could be technical, e.g., due to unequal amounts of cerebellar tissue excised at P1 (which served as a reference for NR2C levels at later stages), we did not further investigate the apparent difference in the mean expression levels. Independently of this, considering the almost complete penetrance of *erbb2/erbb4* inactivation in granule cells of the internal granule layer (IGL) (see preceding paragraph), these data indicate that NRG/ErbB signaling to granule cells is dispensable for the induction of the NR2C subunit observed after the first week of postnatal development.

Development of GABA$_A$-R β2 expression in the cerebellum is indistinguishable from normal in the absence of ErbB2/ErbB4 signaling *in vivo*

GABA$_A$-R β2 is another neurotransmitter receptor subunit strongly expressed in adult cerebellar granule cells (Laurie et al., 1992a) and thought to be regulated by NRG/ErbB (Rieff et al., 1999; Xie et al., 2004). Like for NR2C, expression of this GABA$_A$-R subunit is strongly increased between P6 and P12 (Laurie et al., 1992b). Analysis by qRT-PCR of GABA$_A$-R β2 mRNA in cerebella of wt and *erbb2/erbb4* double knock-out (dKO) mice again did not resolve a difference in the postnatal expression patterns (Fig. 2*B*). Strikingly, compared with NR2C, the postnatal increase of GABA$_A$-R β2 subunit mRNA was less pronounced than would be expected from the strong increase of *in situ* hybridization signals over the same period of development (Laurie et al., 1992a). One reason may be that expression of this subunit is also substantial in other neurons, i.e., Purkinje cells, stellate and basket cells (Laurie et al., 1992b), thus increasing the reference level against which the fold increase is determined.

Cerebellar morphology in the absence of ErbB2/ErbB4 signaling is not distinguishable from normal

We did a number of histological and immunohistochemical stainings to investigate whether the absence of ErbB2/ErbB4 from cerebellar granule cells would affect cerebellar morphology. Qualitative inspection did not reveal abnormalities detectable in cresyl violet-stained sections of the cerebellum in adult (4 months old) mice. Purkinje cells formed a continuous monolayer and the granule cell and molecular layer were of normal thickness (Fig. 3*A*,*B*). This was confirmed by a double-staining of calbindin for Purkinje cells and NeuN for granule cells showing identical results for control and ErbB2/ErbB4 deficient cerebellum (Fig. 3*C*,*D*). Staining for glial fibrillary acidic protein (GFAP) revealed a normal arrangement of Bergmann glia cell processes in the molecular layer (Fig. 3*E*,*F*). Staining of synaptic glomeruli in the granule cell layer and climbing fibers in the molecular layer with the vesicular glutamate transporter 2 (vGlut2) also revealed no differences between control and ErbB2/ErbB4-deficient cerebellum (Fig. 3*G*,*H*). Further staining with antibodies against calretinin, parvalbumin, myelin basic protein, and synaptophysin also yielded identical staining patterns in the ErbB2/ErbB4-deficient cerebellum compared with the wild-type control (data not shown). These data were not quantified using stereological methods, thus they do not exclude the presence of minor quantitative differences between wild type and ErbB2/ErbB4-deficient cerebellum. Similarly, subtle changes in dendritic morphology could not be detected with these stainings. However the availability of antibodies for specific cell types (NeuN, calbindin) and fiber populations (vGlut2) allowed an extensive qualitative evaluation of specific aspects of cerebellar morphology which goes beyond the analysis possible in most other brain regions. These stainings indicate that in the absence of ErbB2/ErbB4 receptors from granule cells the different laminae of the cerebellum form normally, and that synaptic differentiation of the layers proceeds similarly to normal.

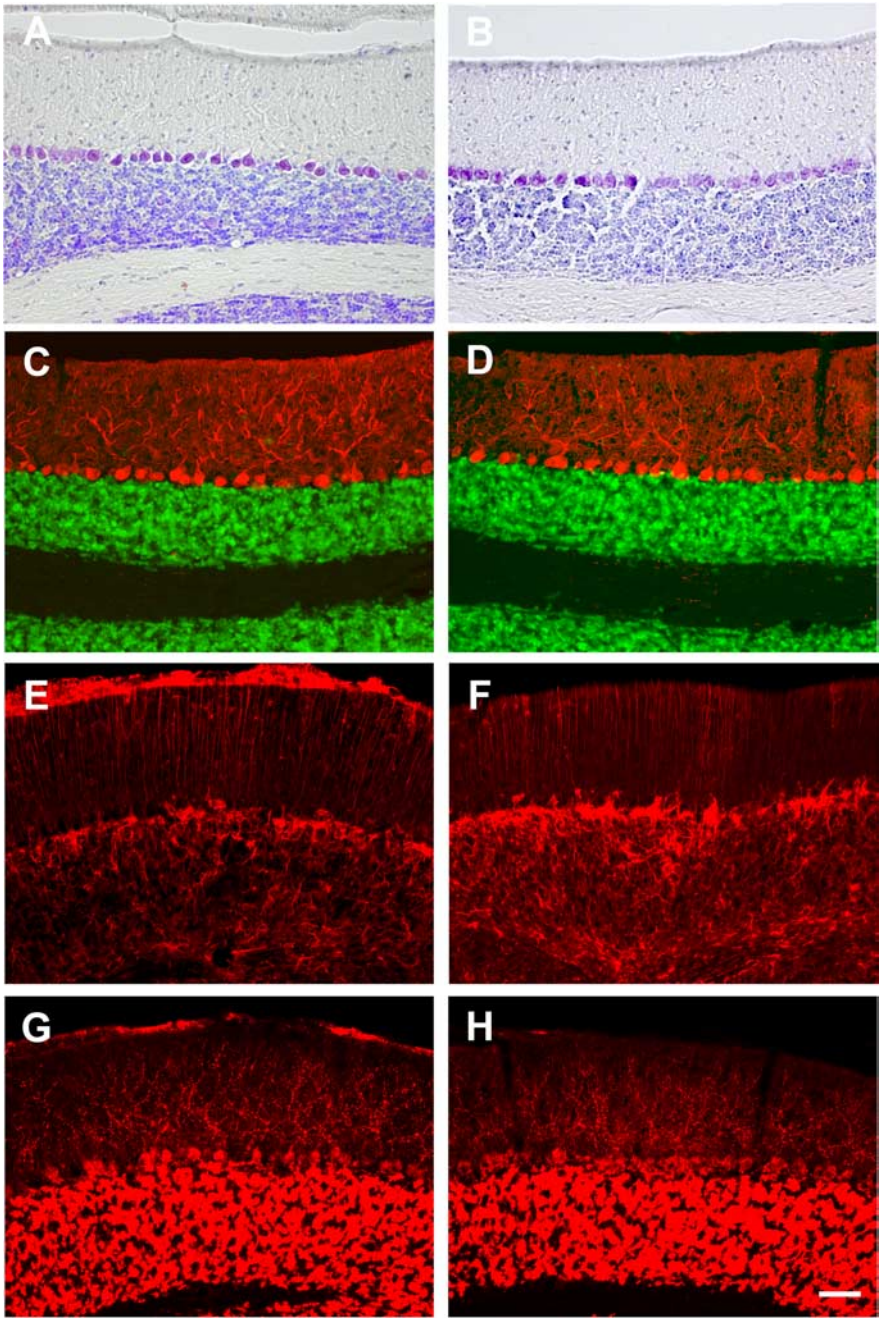

Figure 3. Histological stainings of wild-type (***A***, ***C***, ***E***, ***G***) and *erbb2/erbb4*-deficient (***B***, ***D***, ***F***, ***H***) cerebellum. Scale bar, 50 μm. ***A***, ***B***, Cresyl violet staining of wild-type (***A***) and *erbb2/erbb4*-deficient (***B***) cerebellum. Purkinje cells formed a continuous monolayer, and the granule cell and molecular layer were of normal thickness. ***C***, ***D***, Double immunofluorescence for calbindin (red) to label Purkinje cells and for NeuN (green) to label granule cells. No difference can be seen between control (***C***) and *erbb2/erbb4*-deficient (***D***) cerebellum. ***E***, ***F***, Staining for GFAP revealed an arrangement of Bergmann glia cell processes in the molecular layer, which is not distinguishable in both genotypes. Bright staining on top of the molecular layer in (***E***) represents staining in the meninges (removed in ***F***). ***G***, ***H***, Staining for vGlut2 to reveal synaptic glomeruli in the granule cell layer and climbing fibers in the molecular layer. No difference was detected between wild-type (***G***) and *erbb2/erbb4*-deficient (***H***) cerebellum. Bright staining on top of the molecular layer in (***E***) represents staining in the meninges (removed in ***F***).

Elimination of multiple climbing fiber innervation of Purkinje cells in the absence of ErbB2/ErbB4 signaling is not distinguishable from normal

A process in normal cerebellar development that may be affected by abnormal granule cell function is the elimination of multiple synaptic inputs to Purkinje cells from climbing fibers. Specifically, in early postnatal life Purkinje cells receive synaptic inputs of moderate synaptic strength each from several climbing fibers originating in the inferior olive. By the end of the third postnatal week, this multiple innervation is reduced in most Purkinje cells to one strong input (for review, see Hashimoto and Kano, 2005). The late phase of this multiple climbing fiber elimination is impaired by chronic blockade of NMDA-Rs in the cerebellum (Kakizawa et al., 2000). Purkinje cells in rat are responsive to NMDA only from birth to about P12 (Momiyama et al., 1996), in the

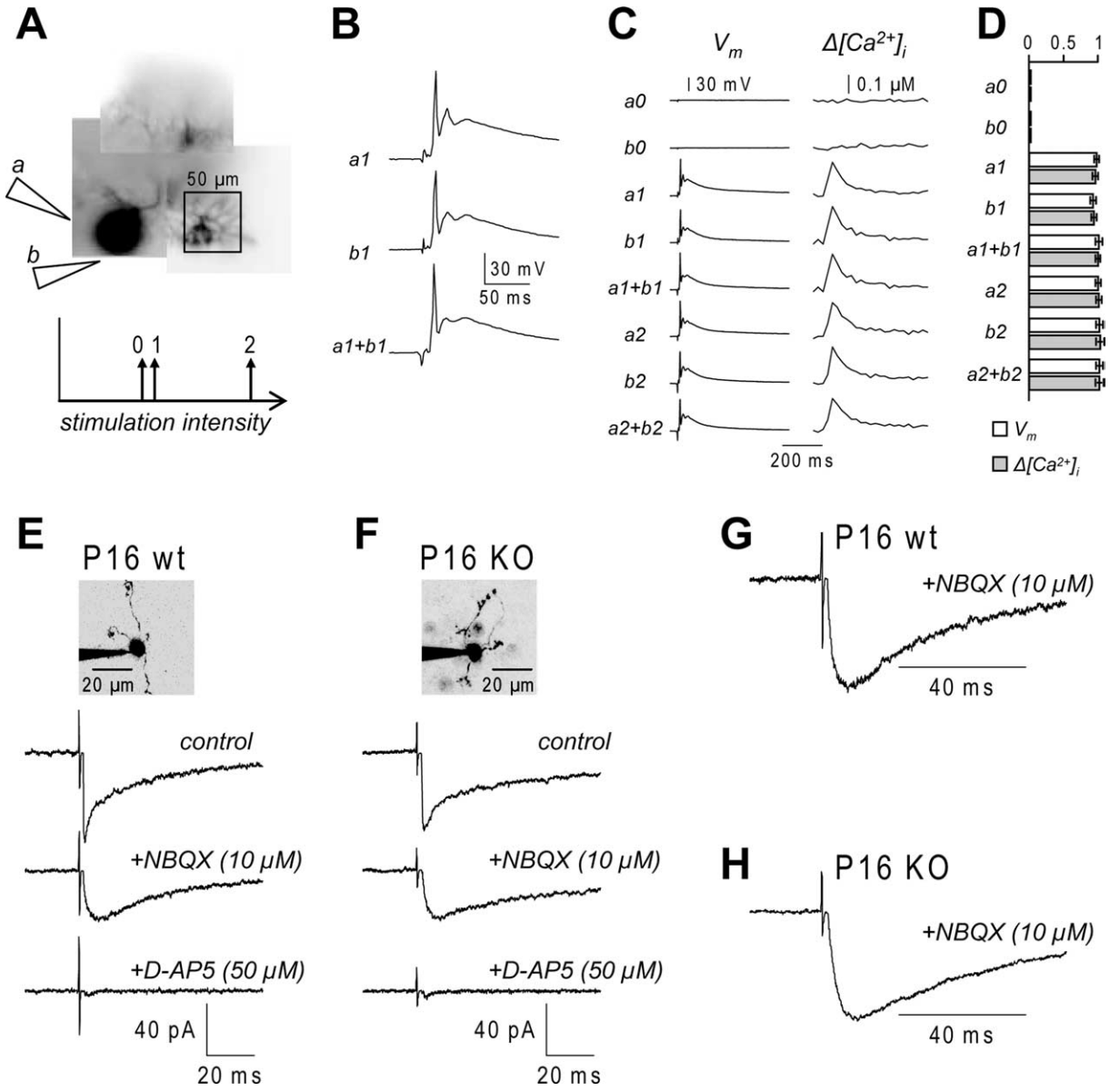

Figure 4. Absence of multiple climbing fiber innervation, normal granule cell morphology, and NR2C expression in *erbb2/erbb4* dKO mice. ***A***, Composite fluorescence image of a Purkinje neuron with the schematic position of two stimulating electrodes (a and b) and a dendritic region used for calcium measurements. Three intensities of stimulation (0, 1, and 2) are illustrated in the relative graph in the bottom; stim 0 below CF activation threshold; stim 1 above CF activation threshold; stim 2 twice stim 1. ***B***, Somatic recordings after CF stimulation with electrodes a and b and with concomitant stimulation of a and b. ***C***, Somatic recordings (left) and associated $\Delta[Ca^{2+}]_i$ signals (right) after CF stimulation at the three different stimulation intensities for electrodes a and b and by concomitant stimulation of a and b at intensities 1 and 2. ***D***, Mean ± SD of EPSP (white bars) and $\Delta[Ca^{2+}]_i$ signals (gray bars) normalized to their means obtained from three cells. The EPSP amplitude was estimated as the average over 25 ms after the peak of the second complex spike (see ***B***). ***E***, Top, Reconstruction of a cerebellar granule cell from a P16 wt animal (top) and MF–EPSCs from another wt cerebellar granule cell at P16 (bottom) in control condition (with 10 μM bicuculline), after addition of 10 μM NBQX, and after further addition of 50 μM AP5. ***F***, Same as ***E*** but from KO cerebellar granule cells at P16. ***G***, NMDA component of the MF–EPSC averaged over five wt cerebellar granule cells at P16. ***H***, NMDA component of the MF–EPSC averaged over seven KO cerebellar granule cells at P16.

mouse recently NMDAR were shown to be present in the adult and make a small contribution to the climbing fiber-evoked current (Renzi et al. 2007). In contrast, NMDA-Rs are abundant at mossy fiber to granule cell synapses throughout the entire CF elimination, the late NMDA-R-dependent phase of this process could be affected via the mossy fiber-granule cell-parallel fiber-Purkinje cell pathway, with an involvement of granule cell NMDA-Rs (Hashimoto and Kano, 2005). If so, impairment of NMDA-R expression and of neurite outgrowth (Rieff et al., 1999; Rieff and Corfas, 2006) in granule cells by ablation of NRG/ErbB signaling could delay the developmental elimination of multiple CF innervation of Purkinje cells.

The probability of persistent multiple CF innervation after the critical developmental period of CF elimination in *erbb2/erbb4*-deficient mutants was tested by measuring CF–EPSPs and the $\Delta[Ca^{2+}]_i$ signals associated with these synaptic signals as described in the methods. Figure 4 shows a representative example of this type of test. In this example, two stimulating electrodes were positioned near the recorded Purkinje neuron as depicted in Figure 4*A* and the threshold stimulation intensity for the activation of a CF–EPSP was found for both stimulating electrodes. Figure 4, *B* and *C*, shows the somatic membrane potential change and the dendritic $\Delta[Ca^{2+}]_i$ signal measured at different stimulating intensities and by concomitant stimulation with the two stim-

ulating electrodes. The stimulating intensities for the two electrodes, a and b, were the minimal current below the threshold for CF activation (a_0 and b_0), the minimal current above the threshold for CF activation (a_1 and b_1) and the current corresponding to the double of minimal current above the threshold for CF activation (a_2 and b_2). The recordings show that neither the amplitude of the CF–EPSP nor the associated $\Delta[Ca^{2+}]_i$ signal changed by doubling the stimulating intensities or by concomitant two-electrode stimulation. The summary results of EPSP and $\Delta[Ca^{2+}]_i$ signal amplitudes from three cells tested in this way are shown in Figure 4*D*. In each cell, both amplitude changes were not statistically significant from their mean ($p > 0.1$, *t* test). No change in the amplitude of CF–EPSC at different stimulating intensities were also found in $N = 5$ Purkinje cell tested without dendritic $\Delta[Ca^{2+}]_i$ signal measurements. Thus, the 95% confidence interval for the probability of multiple climbing fiber innervation is $0 < p < 0.29$, in line with data from wild-type mice (Hashimoto and Kano, 2003; Nishiyama and Linden, 2004). These results indicate that *erbb2/erbb4*-deficient mice do not have persistent multiple CF innervation above the level of wild-type mice after the critical period of development. However, a slight delay in maturation cannot be excluded.

The morphology and the NMDA-mediated currents of cerebellar granule cells in the absence of ErbB2/ErbB4 signaling are not distinguishable from normal

Although the process of CF elimination suggests normal cerebellar development, this evidence does not rule out abnormal dendritic and functional development of cerebellar granule cells.

It has been reported that ErbB receptor signaling regulates dendritic formation (Rieff and Corfas, 2006). Thus, we compared the number of dendrites in wt and *erbb2/erbb4* dKO mice at P16. We filled granule cells with 500 μM Bis-Fura-2 and reconstructed the morphology using two-photon microscopy. Figure 4, *E* and *F* (top), shows granule cells from a wt mouse (Fig. 4*E*) and from a KO mouse (Fig. 4*F*). In all cells analyzed, granule cells had either 3 or 4 dendrites. The mean ± SD for the wt group and the KO group were 3.89 ± 0.93 and 3.94 ± 0.57 respectively, and the two populations were not statistically different (*t* test, $p = 0.89$).

A major feature of the functional maturation of granule cells is the change in the composition of NMDA receptors. In particular, the receptors containing NR2C subunits appear during synaptic pruning (Monyer et al., 1994). In granule cells at P16, the NMDA-mediated component of the MF–EPSC has distinct kinetics which is strikingly different from the NMDA-mediated current lacking NR2C (Ebralidze et al., 1996). Therefore, the measurement of MF–EPSCs in *erbb2/erbb4* dKO mice can give an unambiguous answer on whether or not NR2C are present at P16. Figure 4, *E* and *F* (bottom), shows MF–EPSCs from a wt cell (Fig. 4*E*) and from a KO cell (Fig. 4*F*). In both cases, the NMDA component was isolated from the composite (control) current by adding 10 μM NBQX and blocked by 50 μM AP5. The kinetics of NMDA component in the KO cell was not distinguishable from that recorded in the wild type. Figure 4, *G* and *H*, shows the NMDA-mediated MF–EPSC averaged over five wt granule cells (Fig. 4*G*) and the NMDA-mediated MF–EPSC averaged over seven KO granule cells. The biexponential fit of the wt NMDA components gave a faster decay time constant of 38.8 ± 3.5 ms ($N = 5$) in the range of what reported by Ebralidze et al. (1996). This value was not statistical different (two-populations *t* test, $p = 0.17$) by the faster decay time constant of the KO NMDA components (41.7 ± 3.3 ms, $N = 7$). In contrast, the expected faster decay time constant for NMDA components lacking NR2C is ~25 ms (Ebralidze et al., 1996). Therefore our results demonstrate that NR2C are expressed at P16 in *erbb2/erbb4* dKO mice.

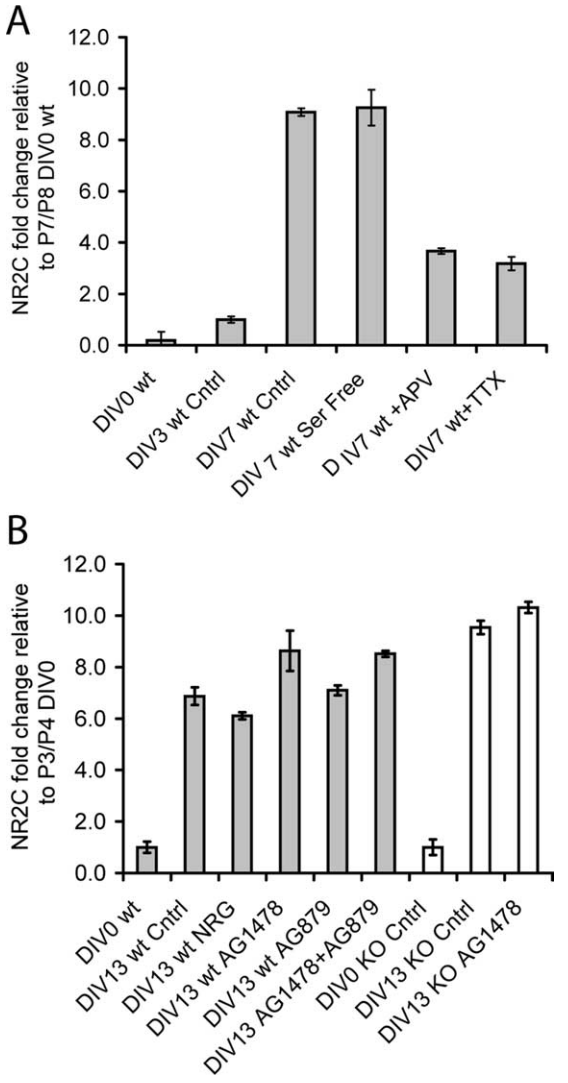

Figure 5. NR2C in P7/P8 (***A***) and P3/P4 (***B***) wt cerebellar slice cultures as fold change relative to that at day 0 *in vitro* (DIV0). ***A***, With TTX (2 μM) or APV (100 μM) treatment there was a reduction in the NR2C expression. Note that the expression pattern was independent of the presence of serum in the medium. ***B***, No significant differences in NR2C expression were observed in cerebellar cultures treated with NRG1β (5 nM), ErbB4 blocker AG1478 (5 μM), ErbB2 blocker AG879 (5 μM), or with a combination of both blockers AG1478 and AG879. Note that NR2C expression was independent of whether cultures were started at P7/P8 or at P3/P4, i.e., before onset of NR2C expression.

Induction of NR2C expression in cerebellar slice cultures in the absence of ErbB2/ErbB4 signaling is not distinguishable from normal

As pointed out above, the inactivation of ErbB2/ErbB4 receptors in granule cells of our mutant *erbb2/erbb4* dKO mice began at around postnatal day 6. To test the possibility that NRG/ErbB-dependent NR2C induction may be initiated before the onset of NR2C expression observed in *erbb2/erbb4* dKO mice, we examined the developmental expression pattern of NR2C mRNA in cerebellar slice cultures in the absence and presence of pharmacological ErbB2/ErbB4 receptor blockers, starting at P7/P8 and at P3/P4. In cultures derived from normal P8 mice, we found a substantial increase in NR2C expression after 1 week (Fig. 5*A*). This increase occurred both in serum containing and serum-free

culture medium and could be partially blocked by addition of TTX or APV to the culture medium (Fig. 5*A*). This indicates that the induction of NR2C expression is at least partially controlled by synaptic activity, as reported by Ozaki et al. (1997). Next, we analyzed NR2C expression in cerebellar slice cultures from P4 mice, which were cultured between 12 and 14 d. Again a strong increase in NR2C expression was found which was present in untreated control cultures and was not altered by addition of 5 nM full-length NRG-β1 extracellular domain to the culture medium (Fig. 5*B*). Furthermore, NR2C induction could not be blocked by addition of ErbB2 antagonist AG879 or ErbB4 antagonist AG1478, nor by both antagonists added together. To further test the independence of NR2C induction of NRG/ErbB2/ErbB4 signaling, we repeated this experiment with cerebellar cultures derived from *erbb2/erbb4*-deficient mice. Even when these cultures were in addition treated with the pharmacological ErbB4 signaling blockers AG1478 we found a normal induction of NR2C expression (Fig. 5*B*). A normal induction of NR2C expression was also found after culturing the ErbB2/ErbB4-deficient slices in serum-free culture medium or with both inhibitors together (data not shown). These findings clearly show that NRG and ErbB2/ErbB4 signaling at early postnatal stages is not required for the later induction of NR2C expression in the cerebellar granule cells.

Discussion

The matching of presynaptically released transmitter substance to the type of postsynaptic receptor expressed is thought to be regulated by signals specific for the type of presynaptic neuron. One example in the CNS is the developmental switch in neurotransmitter receptor expression in postmigratory cerebellar granule neurons that is thought to be induced by the mossy fibers innervating them. These cells express the NR2B receptor subunit at late embryonic and early postnatal stages, but switch to the NR2C subunit beginning in the second postnatal week and extending to the end of the first month (Monyer et al. 1994, Karavanova et al. 2007). Assaying individual granule neurons by single-cell PCR showed that NR2C was expressed specifically in postmigratory granule cells starting at the end of the first postnatal week and continuing until the end of the first postnatal month when all granule cells have completed migration (Durand et al. 2006). At the protein level the switch was resolved electrophysiologically with a similar developmental time course (Feldmeyer and Cull-Candy, 1996). The expression of ErbB receptors in cerebellar granule cells (Ozaki et al. 1998) and of NRG in the neurons synapsing on them is consistent with the idea that this switch in NMDA-R subtype expression is regulated by NRG/ErbB (Corfas et al., 1995; Sandrock et al., 1995). Such involvement was strongly supported by experiments in organotypic slice cultures in which the expression of the NR2C subunit could be induced by application of NRG-1β to the culture medium (Ozaki et al., 1997). Similarly, NRG/ErbB has been implicated in the induction of the $GABA_A$-R β_2 subunit in dissociated cerebellar granule cells in culture (Rieff and Corfas 1999, Xie et al. 2004). Unlike in slice cultures, however, no induction of NR2C expression was found in dissociated cells (Rieff and Corfas 1999). This raises the question what the function of NRG/ErbB signaling in the developmental NMDA-R subtype induction *in vivo* really is. The aim of this study was to examine this question by a genetic approach.

To this end, we ablated ErbB2/ErbB4 receptor expression in the granule cells rather than NRG1 expression in the mossy fiber and Golgi cell projections innervating them. Of the three ErbB receptors, ErbB2, ErbB3 and ErbB4, ErbB2 does not bind ligand and ErbB3 lacks a kinase domain, implying that NRGs can, in principle, signal only through ErbB2/ErbB3, ErbB2/ErbB4 and ErbB3/ErbB4 heterodimers or ErbB4/ErbB4 homodimers (Weiss et al., 1997). Therefore, ablation of ErbB2 and ErbB4 was sufficient to abolish all NRG signaling to the granule cells. This approach offered the additional advantage that compensation by potential products of the related genes *NRG2-4*, all of which signal through ErbB receptors, could be excluded. In our mouse model deletion of the *erbb2* and *erbb4* gene began at P5–P6, i.e., with a time course essentially parallel to the induction of NR2C and the $GABA_A$-R β2 subunits. In these mutants we have examined several aspects of granule cell differentiation and cerebellar integrity. Morphologically, cerebella appeared normal as assayed by immunostainings with markers for specific cerebellar cell types. Neither on Nissl-stained sections nor on immunohistochemical stainings highlighting Purkinje cells and granule cells could any abnormalities be detected. These findings demonstrate that a cerebellum without major abnormalities is formed in *erbb2/erbb4*-deficient mice but they would not exclude the possibility of a certain delay of development in the absence of ErbB2/ErbB4. Immunostaining for the vGlut2 revealed that glomerular synapses were present in apparently normal numbers. Similarly, the developmental elimination of multiple climbing fiber innervations of Purkinje cells, a process thought to be influenced by granule cell function (Hashimoto and Kano, 2005), proceeded normally, thus excluding any major abnormalities in the wiring or neuronal activity of the cerebellum.

In line with this, NR2C and $GABA_A$-R β2 expression developed with a normal time course in *erbb2/erbb4* mutants, and NR2C-mediated current components were unchanged. Recombination of *erbb2/erbb4* was detectable from P4–P6 onwards (Fig. 1), although initially at low levels. Thus, the onset of *erbb2/erbb4* inactivation (P6) (Fig. 1*B*) may appear too close to the induction of NR2C expression (P6) (Karavanova et al., 2007) to fully exclude a role of NRG/ErbB-dependent signaling in this process in the *erbb2/erbb4* dKO mice. It should be noted, however, that both the degree of recombination and the normal development of NR2C mRNA levels in *erbb2/erbb4*-deficient mice are averaged over the entire cerebellum. In contrast, at the level of the GCs in the IGL, ErbB4 s are abolished before synapses are made and NR2C are induced, as indicated by the following observations: (1) GCs migrate as committed precursors from the external granule layer to the IGL where they form dendrites and become innervated by mossy fibers secreting NRG-1. In the rat most granule cells are formed during the second postnatal week, but due to the time required for their migration and the formation of dendrites, few glomerular synapses are formed with mossy fibers before the beginning of the third postnatal week (Altman, 1972). In the mouse, a similar pattern, but with somewhat faster dynamics is observed (Espinosa and Luo, 2008). Therefore, the developmental increase in total DNA recombination (Fig. 1*B*) must be interpreted as recombination in GCs newly arriving in the IGL over a protracted period of time rather than as a delayed recombination of DNA in a constant number of GCs during the time of synapse formation. This interpretation is consistent with the temporal activation pattern of the promoter driving Cre ($GABA_A$-R α6) over the same developmental stages in newly arriving GCs (Zheng et al., 1993). (2) Given the lack of ErbB3 receptors from GCs (Ozaki et al., 1997), and the requirement for ErbB2 to dimerize with ErbB4, the significant reduction of ErbB4 in P16 mutants (Fig. 1*D*) indicates that NRG/ErbB signaling to the GCs is rapidly abolished upon recombination of the gene. Therefore, *erbb4* recombination proceeding from 22% at P6 to

70% at P16 (Fig. 1*B*) should have reduced NR2C expression, if it were dependent on NRG/ErbB signaling. However, at no stage of development did we observe an effect of *erbb2/erbb4* deletion on NR2C and $GABA_A$-R β2 expression (Fig. 2), suggesting independence of their expression of NRG from mossy fibers.

These conclusions *in vivo* are consistent with our data from slice cultures. Specifically, in cultures derived from P3/P4 cerebella, i.e., before synapse formation begins, NR2C expression was induced without addition of exogenous NRG-1β both in serum-containing and in serum-free culture conditions. Furthermore, addition of NRG-1β to the cultures did not affect the expression of NR2C in cultures started at P6/P7, and pharmacological blockers of Erb2 and ErbB4 which were active in a similar *in vitro* system (Li et al., 2007; Pitcher et al., 2008) had no effect on NR2C expression neither in control nor in cultures derived from the *erbb2/erbb4*-deficient mice. This was in contrast to treatments of the cultures with TTX or glutamate receptor antagonists which reduced the induction of NR2C, thus confirming that the culture system used was appropriate for detecting changes in NR2C expression. Combined, these findings suggest strongly that the normal developmental increase in NR2C and $GABA_A$-R expression was not caused by an early activity of NRG/ErbB signaling, i.e., before full *erbb2/erbb4* gene inactivation had taken place.

It should be noted that the present data do not exclude a (redundant) involvement of ErbB-mediated signaling in NR2C expression because the factor inducing it in *erbb2/erbb4*-deficient mice may share downstream signaling cascades with the ErbB-activated pathway. For example, phosphorylation of ERK1/2, the MAPK downstream of NRG/ErbB is also observed at NMJs in ErbB2/ErbB4-deficient muscle (Bezakova and Ruegg, personal communication).

The difference in results between the present *in vivo* and previous *in vitro* experiments may have several explanations. (1) The isolation of slices and of dissociated granule cells deprives the granule cells of their major synaptic input from neurons of the pontine nuclei, a process that may alter their responsiveness to exogenously applied NRGs. (2) As outlined above, the response of cultured granule cells to express NR2C upon treatment with exogenous NRG1 may depend on culture conditions, e.g., the batch of serum used or on the mouse strains used. Specifically, in contrast to a previous study using nominally similar culture conditions (Ozaki et al., 1997), we found a robust spontaneous induction of NR2C under different culture conditions (with high serum or serum-free), although this increase was less pronounced than over corresponding times *in vivo* (8- vs 400-fold during 7 d of culturing). Furthermore, we did not see an effect of purified exogenous NRG1 on NR2C expression, but rather found that NR2C induction proceeded normally in the slice cultures even in the presence of pharmacological ErbB2/ErbB4 blockers or in slice cultures derived from *erbb2/erbb4*-deficient mice. The conflicting findings from culture experiments emphasize the necessity for caution in interpreting such data in the context of developmental mechanisms.

In summary, contrary to previous data from cultured granule cells, the present data have not resolved any evidence for a role of NRG/ErbB signaling in the developmental expression and maintenance of NMDA-R and $GABA_A$-R subtype expression in granule cells in the cerebellum developing *in vivo*.

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