ERBB3-mediated regulation of Bergmann glia proliferation in cerebellar lamination

Anupama Sathyamurthy1, Dong-Min Yin1, Arnab Barik1, Chengyong Shen1,2, Jonathan C. Bean1, Dwight Figueiredo1,2, Jin-Xiong She3, Wen-Cheng Xiong1,2,4 and Lin Mei1,2,4,*

ABSTRACT

Cortical lamination is crucial for the assembly of cerebellar circuitry. In this process, granule neurons (GNs) migrate along Bergmann glia (BG), which are specialized astroglial cells, from the external granule layer to the internal granule layer. However, the molecular mechanisms underlying BG development are not well understood. Here, we show that GFAP::Cre;Erbb3+/− mice, which lack Erbb3 in both radial glia and neurons, exhibit impairments in balance and motor coordination. Cerebellar lamination is aberrant, with misplaced Purkinje neurons and GN clusters. These phenotypes were not observed in Math1::CreERT2;Erbb3+/− mice, where the Erbb3 gene was deleted in GNs, suggesting involvement of non-neuronal Erbb3 in cerebellar lamination. Mechanistic studies indicate that ERBB3 is crucial for the proliferation of BG, which are required for GN migration. These observations identify a crucial role for ERBB3 in cerebellar lamination and reveal a novel mechanism that regulates BG development.

KEY WORDS: Cerebellum, ERBB3, Bergmann glia, Mouse

INTRODUCTION

The cerebellum plays a key role in the neural control of motor movement. It has recently been implicated in higher order processes, including cognition (Buckner, 2013; Fatemi et al., 2012; Manni and Petrosini, 2004). Fundamental to cerebellar function is its unique laminar organization (Apps and Garwicz, 2005). The adult cerebellar cortex has three distinct layers: an outer molecular layer (ML), a middle Purkinje cell layer (PCL) and an internal granule layer (IGL) (Fig. 1A). Cell bodies of granule neurons (GNs) and Purkinje neurons (PNs) reside in the IGL and PCL, respectively, whereas axons of GNs synapse onto dendrites of PNs in the ML. The PCL also contains the soma of Bergmann glia (BG), which extend unipolar processes to contact the pial membrane and are important for cerebellar lamination (Rakic, 1971). During development, granule cell precursors (GCPs) proliferate in the external granule layer (EGL) beneath the glia limitans (formed by Purkinje neurons and GN clusters). These phenotypes were not observed in mice where the Erbb3 gene was deleted in GNs, suggesting involvement of non-neuronal Erbb3 in cerebellar lamination. Mechanistic studies indicate that ERBB3 is crucial for the proliferation of BG, which are required for GN migration. These observations identify a crucial role for ERBB3 in cerebellar lamination and reveal a novel mechanism that regulates BG development.

RESULTS

Loss of ERBB3 in the brain causes motor impairments

To investigate whether ERBB3 plays a role in brain development, we characterized its expression by western blot analysis. As shown in Fig. 1B, ERBB3 was expressed in the brain as early as E16 and plateaued around P20. This temporal expression correlates with cerebellar lamination, which occurs from E15 to P21 (Goldowitz and Hamre, 1998). Embryonic lethality of Erbb3-null mice has precluded the study of the role of ERBB3 in cerbellar lamination (Ericsson et al., 1997; Riethmacher et al., 1997). To circumvent this, we used Cre-Lox-based conditional knockout approach to knock out the Erbb3 gene specifically in the CNS. Floxed Erbb3
mice (Qu et al., 2006) were crossed with GFAP::Cre mice (Zhuo et al., 2001). Cre expression in GFAP::Cre mice occurs as early as E13.5 in precursor cells in the cerebellum that give rise to both GNs and glia. Resulting GFAP::Cre;Erbb3<sup>−/−</sup> mice (hereafter referred to as Erbb3 mutants) were vital at birth and showed reduced expression of ERBB3 in the brain (Fig. 1C; supplementary material Fig. S1A).

At birth, Erbb3 mutant mice appeared normal, had normal brain mass and were indistinguishable from control mice. However, as they aged, mutants showed a smaller increase in brain weight than controls (Fig. 1D). Approximately 90% of mutant mice had significantly smaller brains than controls. There was no difference in body weight between control and mutant mice as late as P90 (Fig. 1E), suggesting that reduction in brain weight was not secondary to body weight reduction. Intriguingly, ataxic gait was observed in 15.6% of the Erbb3 mutants, but not in any of the control mice (supplementary material Movie 1). Based on brain size and gait, mutants could be classified into three groups: group A with normal brain size and gait; group B with smaller brain and normal gait; and group C with smaller brain and ataxic gait (Fig. 1F; supplementary material Fig. S1B).

Next, we determined whether the mutants without obvious gait abnormality were impaired in motor skills. Mice were subjected to elevated beam-walk assays on round or square beams. The amount of time taken to cross the beam and the number of foot slips were quantified. On the first day of trial, control mice crossed the round beam in 15.4±2.1 s (n=9), whereas mutants took significantly longer to cross the beam (Fig. 1G). Both controls and mutants
improved over time, and on day 4, the difference in latency was insignificant. On day 5, when mice were challenged on a narrower, square beam, mutants performed worse than controls (Fig. 1H). These results indicate that Erbb3 mutant mice have impaired motor skills, suggesting that brain regions involved in motor control and coordination might be altered by Erbb3 mutation.

The cerebellum fails to laminate normally in the absence of Erbb3

Motor impairments are often caused by disorders of the cerebellum (Manni and Petrosini, 2004). The findings that GFAP::Cre;Erbb3F/F mutants displayed ataxia and motor defects suggested that the structure and/or function of cerebella were altered. To test this hypothesis, we carried out morphological examination of Nissl-stained cerebellum. At P3, gross anatomy and volume of cerebellum were similar between control and Erbb3 mutant mice (Fig. 2A,B). Although the thickness of the EGL was similar, the fissures that extend between adjacent folia were shorter in mutants (Fig. 2C,D). The EGL and IGL were readily visible at P9 in control and Erbb3 mutant cerebella. However, the EGL and IGL were poorly defined and not easily distinguishable in mutant mice (Fig. 2A). At P30 and P90, most GCPs in the EGL had migrated into the IGL in control mice and thus replaced by the ML. The cerebellum had fully developed folia, and the boundary between the ML and IGL was well defined. In Erbb3 mutants, however, adjacent cerebellar folia appeared to be fused and the boundary between the ML and IGL was ill defined. Ectopic clusters of cells were visible in the ML layer, most likely GNs that failed to migrate into the IGL (Fig. 2A,G, arrow; supplementary material Fig. S2A,E). At P30 and P90, ectopic clusters occupied ~3-4% of the molecular layer in mutants (~0.004% in controls). The cerebella of Erbb3 mutants were smaller at P9, P30 and P90, but not at P3 when the cerebellar cortex starts to develop (Fig. 2A,B,E; supplementary material Fig. S2A-C). In group C Erbb3 mutant mice, which exhibited ataxia (Fig. 1F), cerebellar lamination deficits appeared to be more severe: adjacent folia were not well demarcated. The area occupied by ectopic clusters was greater in group C Erbb3 mutants compared with group B mutants (supplementary material Fig. S2A,B,E).

During cerebellar lamination, GCPs proliferate in the outer region of the EGL and thus are positive for Ki67, a proliferation marker (Li et al., 2013). After mitotic division, GCPs differentiate into immature GNs, which accumulate in the inner EGL and are TuJ1+ (Qu and Smith, 2005). Subsequently, immature GNs migrate to the IGL, where they become mature GNs and express...
NeuN (Weyer and Schilling, 2003). To investigate cellular mechanisms underlying aberrant cerebellar lamination in Erbb3 mutants, P9 cerebella were stained for Ki67, Tuj1 and NeuN. In control mice, Ki67+ GCPs of the outer EGL were in close apposition to the pial membrane that extended into the fissures between adjacent folia (Fig. 3A). Beneath the outer EGL were post-mitotic, Tuj1+ immature GNs (Fig. 3A). By contrast, in Erbb3 mutants, the boundary between the outer and inner EGL was ill defined and Ki67+ GCPs were interspersed among immature GNs in both inner and outer EGL (Fig. 3A). In control mice, mature NeuN+ GNs were confined to the IGL (Fig. 3B). However, in Erbb3 mutants, mature NeuN+ GNs failed to reach the IGL, but instead clustered in the EGL and in areas between the EGL and IGL. These results suggest that ERBB3 plays a role in GN migration. Besides GNs, we examined PNs, another major type of neuron in the cerebellum, which could be labeled by calbindin (Celio, 1990). In control mice, the soma of calbindin+ cells were restricted to the PCL of the cerebellar cortex (Fig. 3C), whereas in mutants, they were not confined to the PCL. Because the Cre in GFAP::Cre mice was expressed in precursor cells that give rise to GNs and glia, but not PNs in the cerebellum (Spassky et al., 2008; Zhuo et al., 2001), PN mislocation may be secondary to cerebellar lamination abnormality stemming from ERBB3 deficiency in other cells.

Neuronal Erbb3 is dispensable for cerebellar lamination

Proliferation and migration of GCPs depend on two cell intrinsic factors: (1) the ability of GCPs to adhere extracellular matrix (ECM) proteins in the basement membrane; and (2) the ability of GNs to extend neurites (Komuro and Yacubova, 2003; Sotelo, 2004). Mutation of genes that regulate GCP adhesion or GN neurite outgrowth result in mislocation of GNs and laminar defects of the cerebellum (Engelkamp et al., 1999; Koirala et al., 2009; Peng et al., 2010). The attachment of GFAP::Cre;Erbb3F/F GCPs to laminin-coated coverslips was similar to that of control GCPs (Fig. 4A,B), suggesting normal adhesion of mutant GCPs to the ECM. Neurite extension was apparently normal in GNs from GFAP::Cre;Erbb3F/F mice, compared with those from controls (Fig. 4C,D). These results suggest that GN misplacement in GFAP::Cre;Erbb3F/F mice may not be due to GCP adhesion or to GN neurite outgrowth. To further test this hypothesis, we selectively ablated Erbb3 in GCPs that give rise to GNs. Math1 (Atoh1 – Mouse Genome Informatics) is a transcription factor that is expressed in GCPs in the rhombic lip and EGL, as well as neurons in nuclei of the hindbrain and midbrain (Ben-Arie et al., 1997). Erbb3F/F mice were crossed with Math1::CreERT2 mice; pregnant dams were injected with tamoxifen at E15, a time when the Cre is induced only in GCPs (Machold and Fishell, 2005). However, Erbb3 deletion (supplementary material Fig. S3A,B) by Math1::CreERT2 did not appear to alter ERBB3 levels.

Fig. 3. Mislocation of granule neurons and Purkinje neurons in the cerebella of Erbb3 mutant mice. Immunostaining of P9 coronal sections from control and mutant mice with indicated antibodies. (A) Ill-defined boundary between outer and inner EGLs. Sections were stained with antibodies against Ki67 (to label GCPs) and Tuj1 (for migrating GNs). Outer EGL is between dashed lines, whereas inner EGL is area between solid and dashed white lines. Solid blue line indicates the pial surface. (B) Presence of mature GNs in the EGL and in areas between the EGL and IGL. Sections were stained with anti-NeuN antibody to label mature GNs. (C) Misalignment of PN soma in mutant cerebellum. Sections were stained with anti-calbindin antibody (to label PNs). Solid white arrowheads indicate the soma of PNs. Pictographs on the right illustrate immunostained cells and cerebellar morphology. Data are representative of results obtained from five mice per group. Scale bars: 100 μm.
in the cerebellum (Fig. 4E), indicating that ERBB3 was not expressed in GCPs or in GNs. In agreement, cerebellar size or morphology in Math1::CreERT2;Erbb3F/F mutants showed no difference from that of control mice after tamoxifen injection (Fig. 4F,G). Unlike GFAP::Cre;Erbb3F/F mutants, ectopic clusters of NeuN+ GNs were not observed in the ML of Math1::CreERT2;Erbb3F/F mice. Instead, NeuN+ GNs were localized in the IGL, as observed in control mice. In addition, staining with GFAP, an intermediate filament protein, showed no difference between tamoxifen-injected control and Math1::CreERT2;Erbb3F/F mice. Expression of Cre was induced as in E. Sections were stained with anti-NeuN antibody. Data are representative of results obtained from eight mice per group. Scale bar: 200 μm. (G) Quantification of cerebellar volume and relative volume occupied by IGL at P60. Every fifth cerebellar section was analyzed to determine the area occupied by the IGL. Area, thickness and spacing of slices were used to estimate volume. n=3 cerebella per group; P>0.05; t-test. (H,I) ERBB3 expression in GFAP+ cerebellar astroglial cells. Cerebellar astrocytes (H) and neurons (I) were cultured from control and GFAP::Cre;Erbb3F/F mice, and at DIV4 (astrocytes) or DIV7 (granule neurons) they were subjected to immunostaining with anti-ERBB3 and anti-GFAP or anti-Tuj1 antibodies. ERBB3 was detected in control GFAP+ glial cells, but not mutant GFAP+ glial cells, control Tuj1+ cells or mutant Tuj1+ cells. White arrowheads indicate cells that express ERBB3 but not GFAP (H) or Tuj1 (I). Scale bars: 50 μm. In B,D,G, data are mean±s.e.m.

Fig. 4. Normal cerebellar lamination in granule neuron-specific Erbb3 mutant mice. (A) Similar number of control and Erbb3 mutant Math1+ cells adhered to laminin. Granule cells from P0 pups (four pups per group) were plated in dishes coated with laminin for 2 h. Shown are images of Math1-staining. Scale bars: 100 μm. (B) Quantification of Math1+ cells in A. n=15 fields for controls and mutants; P=0.17; t-test. (C) Similar neurite outgrowth in cerebellar explants in control and GFAP::Cre;Erbb3F/F mice. Cerebellar explants from P0 mice (four pups per group) were cultured in vitro for 2 days. Shown are images of Tuj1 staining. Scale bars: 100 μm. (D) Quantification of neurite length in A. n=26 and n=25 explants for controls and mutants, respectively; P=0.07; t-test. (E) Similar ERBB3 levels in cerebellar lysates of control and Math1::CreERT2;Erbb3F/F mice. Pregnant dams (at E15) carrying control and Math1::CreERT2;Erbb3F/F embryos were injected with tamoxifen (i.p.), P30 mice were sacrificed and cerebella were isolated for western blot. Similar data were obtained from four controls and four mutants. (F) Normal cerebellar lamination in Math1::CreERT2;Erbb3F/F mice. Expression of Cre was induced as in E. Sections were stained with anti-NeuN antibody. Data are representative of results obtained from eight mice per group. Scale bar: 200 μm. (G) Quantification of cerebellar volume and relative volume occupied by IGL at P60. Every fifth cerebellar section was analyzed to determine the area occupied by the IGL. Area, thickness and spacing of slices were used to estimate volume. n=3 cerebella per group; P>0.05; t-test. (H,I) ERBB3 expression in GFAP+ cerebellar astroglial cells. Cerebellar astrocytes (H) and neurons (I) were cultured from control and GFAP::Cre;Erbb3F/F mice, and at DIV4 (astrocytes) or DIV7 (granule neurons) they were subjected to immunostaining with anti-ERBB3 and anti-GFAP or anti-Tuj1 antibodies. ERBB3 was detected in control GFAP+ glial cells, but not mutant GFAP+ glial cells, control Tuj1+ cells or mutant Tuj1+ cells. White arrowheads indicate cells that express ERBB3 but not GFAP (H) or Tuj1 (I). Scale bars: 50 μm. In B,D,G, data are mean±s.e.m.

in the cerebellum (Fig. 4E), indicating that ERBB3 was not expressed in GCPs or in GNs. In agreement, cerebellar size or morphology in Math1::CreERT2;Erbb3F/F mutants showed no difference from that of control mice after tamoxifen injection (Fig. 4F,G). Unlike GFAP::Cre;Erbb3F/F mutants, ecotropic clusters of NeuN+ GNs were not observed in the ML of Math1::CreERT2;Erbb3F/F mice. Instead, NeuN+ GNs were localized in the IGL, as observed in control mice. In addition, staining with GFAP, an intermediate filament protein, showed no difference between tamoxifen-injected control and Math1::CreERT2;Erbb3F/F mutants (supplementary material Fig. S3C), suggesting that the Erbb3 gene in GCPs or GNs was not required for BG development or maintenance. Together, these observations indicate that lack of the Erbb3 gene in GCPs or GNs has no effect on cerebellar cortical lamination.

Glial scaffolding abnormalities in Erbb3 mutant mice

The above results suggest that the phenotypes in Erbb3 mutants may be due to lack of Erbb3 in glial cells in the cerebellum. Cerebellar glial cells, including BG, are derived from glial precursors in the ventricular zone of the fourth ventricle and express GFAP (Bignami and Dahl, 1973; Yamada and Watanabe, 2002). We determined whether ERBB3 was expressed in astroglial cells of the cerebellum. Cerebellar astrocytes (H) and neurons (I) were cultured from control and GFAP::Cre;Erbb3F/F mice, and at DIV4 (astrocytes) or DIV7 (granule neurons) they were subjected to immunostaining with anti-ERBB3 and anti-GFAP or anti-Tuj1 antibodies. ERBB3 was detected in control GFAP+ glial cells, but not mutant GFAP+ glial cells, control Tuj1+ cells or mutant Tuj1+ cells. White arrowheads indicate cells that express ERBB3 but not GFAP (H) or Tuj1 (I). Scale bars: 50 μm. In B,D,G, data are mean±s.e.m.
During development, unipolar BG extend processes to the pial surface; immature GNs migrate on these processes from the EGL to the IGL (Rakic, 1971). Genetic ablation of cerebellar astrocytes, including BG, at P1 leads to small cerebellar, GN and PN mispositioning and severe ataxia (Cui et al., 2001; Delaney et al., 1996). Because Erbb3 mutants exhibited similar phenotypes and because ERBB3 is expressed in cerebellar astroglial cells, we characterized BG morphology in Erbb3 mutants. Cerebellar coronal sections were stained with anti-GFAP antibody to visualize BG cytoskeleton. In cerebella of P9 control mice, GFAP+ astrocytes were present in the white matter (Fig. 5). BG, which are located in the PCL, extended parallel processes to the pial surface, forming glial scaffolds. Their endfeet were in close apposition to the pial surface, forming glia limitans (Fig. 5, arrowhead). However, in Erbb3 mutants, there was a significant reduction in GFAP staining in the white matter. Remarkably, the glial scaffolds were severely disorganized and failed to form glia limitans at P9. Similar deficits were observed in P90 mutant mice (supplementary material Fig. S4).

The disorganized glial scaffolds may be caused by the inability of Erbb3 mutant BG to form a scaffold. Alternatively, it could be due to reduced numbers of BG. To determine whether Erbb3 mutant cerebellar astroglial cells were able to form radial glial scaffold, we analyzed their morphology in culture by performing BLBP immunostaining. BLBP is expressed in most cerebellar astrocytes, including Bergmann glia (Feng et al., 1994). Under normal conditions, the percentage of control and mutant cells that displayed radial glial morphology (supplementary material Fig. S5A, yellow arrowhead) was similar. A previous study has shown that NRG1 can induce radial glial morphology in cultured cerebellar astrocytes (Rio et al., 1997). In control cells, NRG1 treatment induced radial glial morphology in a dose-dependent manner. This effect of NRG1 was not abolished in Erbb3 mutant cerebellar astroglial cells (supplementary material Fig. S5A). Together, these results suggest that ERBB3 is dispensable for scaffold formation by cerebellar astroglial cells.

**BG reduction in Erbb3 mutant cerebellum**

To determine whether inadequate scaffolding was due to reduced number of BG, we performed BLBP immunostaining to visualize BG at different stages of BG development. Around E15, there is an acceleration in BG migration from the ventricular zone of the fourth ventricle, where they are generated, to final destination in the PCL (Yamada and Watanabe, 2002). Once BG reach the PCL, they proliferate in the growing PCL. This proliferative activity of BG during the perinatal period is important for cerebellar development (Delaney et al., 1996). In order to determine whether embryonic generation and migration of BG was normal in mutants, we performed BLBP immunostaining at E18.5. As shown in Fig. 6A, the number of BLBP+ cells in the PCL was similar between controls and mutants at E18.5, suggesting that generation and migration of BG was not altered in mutants. In P9 cerebellar slices of control mice, the soma of BG were present in the PCL layer. They appeared to extend processes towards the pial membrane (Fig. 6B). However in P9 Erbb3 mutants, BG soma were conspicuously absent in the PCL, and there were no well-defined glial scaffolds. Similar results were obtained by immunostaining for S100β, a cytoplasmic protein that is expressed in cerebellar glial cells, including cerebellar astrocytes and BG (Fig. 6C). These results indicate that the number of BG cells was reduced in Erbb3 mutant cerebellum. Next, we isolated cerebellar astroglial cells from control and mutant cerebella, and analyzed their ability to expand in culture (Fig. 6D,E). The number of mutant cerebellar astroglial cells was only 50% of control at DIV12. Together, these data suggest that Erbb3 ablation impairs the expansion of cerebellar glial cells.

Reduction in astroglial number could either be due to increased apoptosis or to reduced proliferation of astroglial cells. To determine whether Erbb3 ablation alters the apoptosis of cerebellar astroglial cells, we performed TUNEL and GFAP staining to visualize apoptotic astroglial cells. As shown in supplementary material Fig. S5B, TUNEL-labeled GFAP+ cells were similar between control and mutant mice, suggesting little effect of Erbb3 ablation on apoptosis of cerebellar astroglial cells. We next determined whether ERBB3 is
necessary for astroglial proliferation. Cerebellar astroglial cells were subjected to staining with antibodies against GFAP and Ki67, indicating that the proliferation of cerebellar astroglial cells requires ERBB3. Together, these data suggest that reduced number of mutant Erbb3 cerebellar astroglial cells results from impaired proliferation, but not from increased cell death.

The number of TUNEL-labeled cells in the PCL was similar between control and Erbb3 mutant mice (Fig. 7A), in agreement with the notion that the reduction in the number of BG in mutants was not due to compromised BG survival. To determine whether BG proliferation was reduced in vivo in Erbb3 mutant mice, we determined the number of proliferating, Ki67+ glial cells in the PCL at different ages. In the PCL, BG are mitotic and thus Ki67+, whereas PNs are post-mitotic and should not be labeled by Ki67. As shown in Fig. 7B,C, the number of Ki67+ BG in the PCL was significantly reduced in the mutants as early as E18.5 (27.7±4.6% of controls). These Ki67+ cells in the PCL also expressed nestin, an intermediate filament protein expressed in cerebellar astroglial cells, including BG (Supplementary material Fig. S5C). At P0.5, proliferating BG were further reduced in the mutants (49.2±2.8% of controls) (Fig. 7B,C). Together, these results support a model where cerebellar BG require ERBB3 for proliferation, and that the absence of ERBB3 leads to cell-autonomous impairments in proliferation that in turn result in cerebellar lamination defects.

**DISCUSSION**

Our study provides genetic evidence for a role for ERBB3 in cerebellar development. Erbb3 mutant mice exhibited deficits in motor coordination. Cerebellar lamination was aberrant in GFAP::Cre;Erbb3F/F mice, which lack Erbb3 in both neurons and glia of the cerebellum. Both GNs and PNs were misplaced in the mutant mice (Figs 1-3). Moreover, GNs failed to migrate into the IGL and formed ectopic clusters in the ML. The boundary between the IGL and the ML was indiscernible. These observations demonstrate that ERBB3 is necessary for cerebellar lamination. Mechanistically, ablation of the Erbb3 gene in GNs (by Math1::CreERT2) had no effect on GN migration (Fig. 4). Furthermore, although CRE is not expressed in PN precursors in GFAP::Cre mice (Anthony and Heintz, 2008; Zhuo et al., 2001), PN soma were misaligned in mutant cerebellum. These results suggest that loss of Erbb3 in cells other than GNs and PNs contributes to the lamination defects in GFAP::Cre;Erbb3F/F mice. Moreover, the number of Ki67+ cells was reduced in the PCL of GFAP::Cre;Erbb3F/F mice, suggesting that BG proliferation was reduced (Fig. 7). Finally, ERBB3 was expressed in cerebellar astroglial cells and was required for their proliferation in culture (Figs 4 and 6). These observations support a working model where cerebellar BG require ERBB3 for proliferation. Diminished BG proliferation in the absence of ERBB3 causes reduction and disorganization of BG scaffold, that is required for GN migration and cerebellar lamination.

ERBB3 is important for morphogenesis of different organs and tissues, including the heart, pancreas, adrenal gland, mammary...
gland and peripheral nervous system (PNS) (Erickson et al., 1997; Jackson-Fisher et al., 2008; Riethmacher et al., 1997). Most Erbb3-null embryos die at E13.5 due to cardiac defects and display defects in the midbrain/hindbrain region, including the cerebellum (Erickson et al., 1997). Erbb3-null mice that are born die soon after birth from death of sensory and motor neurons, presumably due to impaired survival or proliferation of Schwann cell precursors (SCPs) (Lyons et al., 2005; Riethmacher et al., 1997). ErbB3 has been implicated in myelination in the PNS as well as the CNS (Brinkmann et al., 2008; Makinodan et al., 2012). Nrg1-null embryos exhibit cardiac defects, similar to Erbb3-null embryos; mice lacking Nrg1 in motor neurons display motor neuron death, and myelination defects in the PNS (Brinkmann et al., 2008; Erickson et al., 1997; Yang et al., 2001). Because of embryonic lethality of Nrg1-null mutation, understanding of NRG1-ERBB signaling in CNS development came mostly from in vitro and explant studies. In one model, NRG1 promotes radial migration of neurons in the cerebellum and cerebrum by stimulating ERBB kinases in BG and radial glial cells, respectively (Anton et al., 1997; Neurons in the cerebellum and cerebrum by stimulating ERBB explant studies. In one model, NRG1 promotes radial migration of the two ERBBs with active kinase domains and whose kinase activities can be stimulated by NRG1 (Holmes et al., 1992; Plowman et al., 1993), by GFAP::Cre: did not reveal morphological abnormalities in the cerebellum and cerebrum (Barros et al., 2009). These observations suggest that the NRG1 signaling pathway may not be necessary for cortical lamination. In agreement, knockout of Nrg1 by GFAP::Cre had no effect on cerebellar lamination (data not shown).

In addition to NRG1, two other NRG proteins, NRG2 and NRG6, bind to ERBB3 and are expressed in the cerebellum (Aono et al., 2000; Busfield et al., 1997; Carraway et al., 1997; Juttner et al., 2005; Kinugasa et al., 2004). Moreover, recent data suggest that ERBB3 possesses some intrinsic kinase activity for auto-phosphorylation, albeit 1000-fold less active than EGFR (Shi et al., 2010). It is unknown whether ERBB3 is regulated by a NRG ligand. Nrg2- and Nrg6-null mice are viable without apparent deficits in cerebellar lamination (Britto et al., 2004; Juttner et al., 2005), probably due to functional redundancy of NRGs. The existence of multiple NRG ligands for ERBB3 suggests functional redundancy, which adds a layer of complexity in delineating the role of each NRG in neural development. Adding to the complexity is the fact that ERBB3 may dimerize with ERBB2 and ERBB4 (Berger et al., 2004). However, previous studies have shown that ERBB2 and ERBB4 are dispensable for cerebellar lamination (Barros et al., 2009; Gajendran et al., 2009). Therefore, ERBB3 regulation of cerebellar lamination may be independent of other ERBBs. Double or triple mutation of different NRGs may be necessary to unravel the role of NRGs in cerebellar development.

PI3K activation has been implicated in ERBB3-dependent proliferation and survival of various cancer cells (Cook et al., 2011; Smimova et al., 2012; Young et al., 2013). ERBB3 contains seven binding motifs for PI3K, in contrast to one in ERBB4 and none in EGFR and ERBB2 (Schulze et al., 2005; Soltov et al., 1994). Deletion of Pten, a negative regulator of PI3K-AKT signaling, results in cerebellar lamination deficits (Marino et al., 2002; Yue et al., 2005), suggesting that normal levels of PI3K-AKT activity are important for cerebellar lamination. GRB2, an adaptor protein, can bind to two phosphorylated tyrosine residues in the ICD of ERBB3 and activate the MAPK signaling cascade, which has been implicated in proliferation of glial cells (Fischer et al., 2009; Schulze et al., 2005; Zhang and Liu, 2002). Moreover, the adaptor CRK has high affinity for pY1276 in the ICD of ERBB3. Loss of CRK and/or reduced phosphorylation of CRK leads to abnormal lamination of the cerebellar cortex (Park and Curran, 2008; Jones et al., 2006;
Qiu et al., 2010). Intriguingly, levels of pAKT, pERK and pCRK in cerebellar lysates were similar between control and Erbb3 mutant pups (supplementary material Fig. S6), suggesting that these signaling pathways may be dispensable for Erbb3-mediated BG proliferation.

Previous studies suggest that proliferation and survival of astrocytes and neural precursors requires FGF (fibroblast growth factor) signaling (Kang and Song, 2010; Morrison and de Vellis, 1981). Interestingly, Erbb3 is required for FGFR2-dependent proliferation of cancer cells (Kunii et al., 2008). Erbb3 may act together with FGF signaling to regulate cerebellar laminatin. In support of this notion are observations that conditional knockout of Fgfr2 or double knockout of two FGF receptors (FGFR1 and FGFR2) causes cerebellar deficits similar to those observed in Erbb3 mutants (Lin et al., 2009).

In conclusion, our study establishes an essential role for Erbb3 in cerebellar laminatin, identifying a novel function of Erbb3 in BG proliferation, an important step during cerebellar development. Our study demonstrates that cerebellar astroglia, including BG, require Erbb3 for proliferation. In the absence of Erbb3, BG fail to proliferate and thus cause insufficient scaffolding for GN migration, a process that is essential for cerebellar laminatin.

MATERIALS AND METHODS

Generation of mice

Erbb3<sup>F/F</sup> (SV129/C57BL) mice were crossed with GFAP::Cre (FVB/J) or Mathl::CreER<sup>T2</sup> (FVB/J) mice (Machold and Fishell, 2005; Qiu et al., 2006; Zhuo et al., 2001). All mice were housed in a room at 23°C in a 12 h light/dark cycle with access to food and water ad libitum. Math1::CreER<sup>T2</sup> was activated by injecting pregnant dams intraperitoneally (i.p.) once with tamoxifen at 4 mg/35 g mouse. PCR-based genotyping was performed on tail genomic DNA. The following primers were used:

- forward, TGTTTGTGAAATGTGGACTTTACC; reverse, CCAACCC-
- forward, GGCAGGCATGTTGACTTCACTTGT; reverse, GCGGTCTG-

Tail genomic DNA. The following primers were used:

- forward, ACTCCTTCATAAAGCCCTCG; reverse, ATCACTCGTTGCATCGACCG; forward, GGGAGCACTGTGACTCCTTG; reverse, CCAACCTTCTCTCAGATAGG; wild-type Erbb3 allele (forward, GTGGAAACAGCATTGCTGTCACTT; reverse, GTGAAACAGCATTGCTGTCACTT).

Experimental procedures were approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Western blotting

Western blotting was performed as described previously (Barik et al., 2014; Shen et al., 2013; Yin et al., 2013a). Brain tissues were homogenized in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% SDS, 1 mM PMSF, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT and protease inhibitor cocktail. Debris was cleared by centrifuging samples at 12,000 g for 20 min at 4°C. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) that were blocked in Tris-buffered saline (TBS) containing 0.1% Tween and 5% skim milk for 1 h at room temperature. Blocked membranes were incubated in primary antibody overnight at 4°C and then in HRP-coupled secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence. The following dilutions of primary antibodies were used: rabbit anti-ErbB3 (1:500, sc-285, Santa-Cruz), rabbit anti-ERBB3 (1:100, 12708S, Cell Signaling), mouse anti-α-tubulin (1:5000, sc-23948, Santa Cruz), mouse anti-CRK (1:1000, 610035, BD Biosciences), rabbit anti-phospho CRK (1:1000, 3491, Cell Signaling), rabbit anti-AKT (1:1000, 9272, Cell Signaling), rabbit anti-P-AKT (1:1000, 9271, Cell Signaling), rabbit anti-ERK (1:1000, 9102, Cell Signaling), rabbit anti-p-ERK (1:1000, 4377, Cell Signaling) and mouse anti-β-actin (1:1000, A1978, Sigma).

Cell culture

Granule cells and astroglia cells were purified as previously described (Hatten, 1985). Briefly, cerebellum were isolated from P0 mouse pups in ice-cold phosphate-buffered saline (PBS) (Corning). After the meninges were removed, cerebella were cut into small pieces and incubated in PBS containing 0.25% trypsin (Corning) and 0.1% deoxyribonuclease (Sigma) at 37°C for 40 min. Trypsin was inactivated by incubation with DMEM (Corning) containing 10% fetal bovine serum (FBS) (Life Technologies) and penicillin-streptomycin (PS) (HyClone). The mixture was centrifuged at 3000 rpm (500 g) for 5 min. The pellet was resuspended in PBS and used for preparation of GCPs (see below) and astroglial cells. For astroglial culture, the suspended pellet was loaded on top of a two-layer Percoll (Sigma) gradient (35% Percoll and 60% Percoll in PBS) and centrifuged at 3000 rpm (1550 g) for 5 min. After centrifugation, astroglial cells were collected from the top of the gradient, and recentrifuged and resuspended in DMEM containing 10% FBS and PS. The mixture of cells was passed through a 70 µm nylon filter (Falcon) to obtain a single cell suspension and pre-plated twice on uncoated 100 mm dishes (Bio-lite) for 20 min each to remove contaminant fibroblasts. Media containing unattached cells were aspirated and plated in triplicate on 12-well plates containing coverslips coated with 75 µg/ml of poly-L-lysine (Sigma) in PBS. After 4 h, fresh DMEM containing 10% FBS and PS was added to the cells. Half the media was replaced with fresh media once every 3 days.

GNCs were cultured as described previously (Lee et al., 2009). Briefly, GNCs were collected from the interface between the Percoll gradients, and recentrifuged and resuspended in DMEM containing 10% FBS. The mixture of cells was passed through a 40 µm nylon filter to obtain a single cell suspension and pre-plated twice on uncoated 100 mm dishes for 20 min each to remove contaminant fibroblasts. Media containing unattached cells were aspirated and plated in triplicate on 12-well plates containing coverslips coated with 500 µg/ml of poly-L-lysine (Sigma) in PBS. After 4 h, plating medium was replaced by neurobasal medium supplemented with 200 µM L-glutamine, B27 and PS. Half of the media was replaced with fresh media once every 3 days.

To prepare GCPs for adhesion assay, the cell pellet was resuspended in serum-free DMEM containing PS and passed through a 40 µm nylon filter to obtain a single cell suspension. Cells were pre-plated twice on uncoated 100 mm dishes for 20 min each to remove contaminant fibroblasts and plated in triplicates on 12-well plates containing coverslips coated with 2 µg/ml laminin (L2020, Sigma). Cerebellar microproliferats were cultured as described previously (Fischer et al., 1986), with modifications. P0 cerebella were stripped of meninges and deep cerebellar nuclei, and the remaining cortical tissue was minced and suspended in PBS. The suspension was spun at 1000 rpm (150 g) and the pellet was washed twice with DMEM/F12 media containing PS and plated on coverslips coated with 100 µg/ml poly-L-lysine and 10 µg/ml laminin, and maintained in DMEM/F12 media containing 20 µg/ml insulin, 1 mg/ml bovine serum albumin (BSA), 30 mM glucose, 1.8 mM glutamine, 24 mM sodium bicarbonate and PS. Coverslips were washed twice with PBS, fixed in 4% PFA and processed for immunostaining (see below).

Immunostaining

Immunostaining was performed as described previously (Yin et al., 2013a). Anesthetized mice were perfused transcardially with 4% PFA in PBS and tissues were fixed in 4% PFA at 4°C for 12 h. Frozen brain blocks were sectioned to yield 35-40 µm thick floating coronal slices. Slices were permeabilized and blocked in PBS containing 5% BSA (Sigma) and 0.3% Triton X-100 (Sigma), and incubated with primary antibodies at 4°C overnight. Primary antibodies were prepared in PBS containing 0.3% Triton X-100 by the following dilutions: mouse anti-NeuN (1:500, MAB377, Millipore), rabbit anti-ErbB3 (1:100, 12708S, Cell Signaling), mouse anti-GAP-43 (1:500, MAB360, Chemicon), rabbit anti-S100 (1:500, z0311129-2, Dako), rabbit anti-BLB (1:500, ab32423, Abcam), rabbit anti-KC d (1:500, RM-9102-S1, Abcam), rabbit anti-Tuj1 (1:500, MMS-435P, Covance), rabbit anti-parvalbumin (1:500, PV-25, Swant) and mouse anti-ki67 (1:500, D28K-300, Swant). Slices were washed three times in PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Slices were washed three times in PBS and mounted with VectaShield mounting medium (H-1200, Vector laboratories). TUNEL staining of fresh frozen brain sections was performed using In Situ Cell
Death Detection Kit (Fluorescein) (11684795910, Roche), following the manufacturer’s instructions. Images were captured using a confocal LSM 510 NLO system (Zeiss) and processed using FIJI software.

Elevated beam-walk assay
Beam-walk assay was performed as described previously (Carter et al., 2001; Luong et al., 2011). On days 1-4, mice (3 months old) were subjected to testing on a round beam (12 mm in diameter, 1 m in length, 50 cm from the ground) (twice on days 1-2 and once on days 3-4) and latency to cross the beam was measured. On day 5, mice were tested on a square beam (5 mm in width, 80 cm in length, 50 cm from the ground). Both latency and foot slips were measured. During the tests, mice occasionally stalled while crossing the beam. They were encouraged to cross the beam by gentle prodding. However, the stalling time was not included in the readout. If a mouse was unable to cross the beam, 60 s was entered for a particular trial.

Quantitative RT-PCR analysis
Total RNA was isolated from mouse brains using Trizol (Invitrogen) and purified using the RNasey Mini Kit (Qiagen). Equal amounts of total RNA (4 µg) were reverse-transcribed by random hexamer primers using SuperScript III reverse transcriptase (Invitrogen). cDNA was analyzed by quantitative real-time PCR (qPCR) in triplicate using SYBR Green/ROX (Fermentas) on a Chromo 4 (Bio-Rad). mRNA levels were normalized to Gapdh that was assayed simultaneously on the same reaction plate. The following primers were used: Erbb3 forward AGTGCGAATTGGAGCTCTG and reverse CTCCAGGTACCATCAGACAC; Gapdh forward AGCTG- GGTGGACGTGATTTG and reverse GGAGTCGGTGTAGGCAACA. Products were resolved by agarose gel electrophoresis.

Statistical analysis
Statistical analyses were performed using GraphPad prism software. Data are presented as mean±s.e.m. and analyzed using Student’s t-test, one-way ANOVA or two-way ANOVA, as appropriate. Differences among groups were considered significant if P<0.05. P-values are denoted by asterisks: *P<0.05; **P<0.01; ***P<0.001.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary material
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