

SHORT COMMUNICATION

ErbB receptor signalling regulates dendrite formation in mouse cerebellar granule cells *in vivo*

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Abstract

The formation of morphologically and functionally mature dendrites is a key event in neuronal maturation and the establishment of functional neuronal networks, but the signals that regulate mammalian dendritic development remain poorly understood. Here we show that the erbB receptor signalling pathway, which modulates expression of several neurotransmitter receptors, also regulates dendritic development of cerebellar granule cells in the intact cerebellum. These results suggest that neuregulin–erbB signalling may control a program of postsynaptic development, from initiating dendrite morphogenesis to the formation and maturation of the postsynaptic apparatus.

Introduction

The formation of dendrites is a key event in neuronal maturation and the establishment of neuronal networks. Development of functional dendrites involves not only the elaboration of dendritic arbors but also the formation of the postsynaptic apparatus necessary for synaptic transmission. There is growing evidence that both morphological and synaptic maturation of dendrites are strongly influenced by signals derived from the presynaptic cells, including extracellular signalling molecules and electrical activity (Scott & Luo, 2001; Whitford *et al.*, 2002; Van Aelst & Cline, 2004). While several molecules that regulate diverse aspects of dendritic development have been identified, the extent to which morphological and molecular dendritic maturation are coordinated remains undetermined. In previous studies we found evidence suggesting that the growth factor neuregulin 1 (NRG1) and its tyrosine kinase receptors, the erbB receptors, regulate several aspects of postsynaptic development of cerebellar granule neurons. *In vitro* studies have shown that NRG1 induces the expression of γ -aminobutyric acid-A (GABA_A; Rieff *et al.*, 1999; Xie *et al.*, 2004) and *N*-methyl D-aspartate (NMDA) receptors (Ozaki *et al.*, 1997) in granule cells, indicating that this pathway could be involved in the formation of the postsynaptic apparatus of granule cells. Interestingly, we also found that NRG1 induces neurite outgrowth by these neurons, indicating that it could also regulate dendritic morphogenesis. Furthermore, the pattern of expression of NRG1 and erbB4 in the intact cerebellum provided further support for this possibility. When developing granule cells complete their migration and reach the internal granule layer of the cerebellum, they begin to receive synaptic inputs and start expressing the NRG1 receptor erbB4 (Rieff *et al.*, 1999) while the sources of their synaptic inputs, the Golgi cells and

mossy fibers, express NRG1 (Corfas *et al.*, 1995). In this study we tested whether NRG1-erbB signalling is involved in the formation of granule cell dendrites *in vivo*. We show that, when erbB signalling is blocked, the number of granule cell dendrites is significantly reduced, suggesting that NRG1-erbB signalling may control a program of postsynaptic development, from initiating dendrite morphogenesis to regulating postsynaptic apparatus maturation.

Materials and methods

Virus production

The 2.2-kb dominant negative erbB4 receptor (DN-erbB4) cDNA (Rio *et al.*, 1997) was cloned directionally into the replication-incompetent retroviral vector pLIA (Bao & Cepko, 1997). This vector expresses exogenous genes under the control of the MMLV long-terminal repeat followed by an internal ribosomal entry site (IRES) fused in frame to the human placental alkaline phosphatase (AP) gene (Bao & Cepko, 1997). Viral production, concentration and titring were carried out as described (Pear & Cepko, 1996).

Virus injection

Postnatal day (P)1 CD-1 mice were anaesthetized with cold, and 2–3 μ L of concentrated virus solution was injected by pressure into three sites in the cerebellum (at the midline, and a few millimetres to either side of the midline) using borosilicate glass electrodes (World Precision Instruments). The injections were targeted to the most superficial layer of the cerebellum in order to infect dividing granule cell precursors within the external granule cell layer (EGL). Three litters of mice were used; in each one half the pups were injected with LIA virus (identified by a tail clip) and half with LIA-DN-erbB4. This study was approved by the Animal Care and Use Committee of Harvard Medical School Children's Hospital.

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Histology

Cells in culture were fixed in 4% paraformaldehyde for 20 min at room temperature. Mice were killed with CO₂ and fixed by cardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cerebella were dissected, embedded in OCT compound (Tissue-Tek) and cryosectioned parasagittally at 50 µm thickness. For erbB4 immunostaining, cells or tissue sections were blocked in 3% BSA and 0.1% Triton-X in PBS for 1 h at room temperature, and then incubated in the same buffer containing antihuman erbB4 antibody (HER-4 Ab-1; 1 : 200; Neomarkers, Fremont, CA, USA) overnight at 4 °C. Cells or slides were then washed in PBS (three times, 10–15 min each), incubated with cyanine 3 (Cy3) antimouse secondary antibody at 1 : 500 in 3% BSA for 1 h at room temperature, and washed again in PBS. AP staining of cells in culture or tissue sections was carried out as described previously (Fields-Berry *et al.*, 1992). Briefly, after fixation and rinsing, tissues were heat-inactivated to 65 °C for 30 and 50 min for cells and cerebellar sections, respectively, to eliminate endogenous phosphatase activity. Then, the infected cells were stained overnight with X-Phos staining solution containing X-Phos staining buffer (in mM: Tris-HCl, pH 9.5, 100; NaCl, 100; MgCl₂, 50), 100 µg/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Sigma) and 500 µg/mL nitroblue tetrazolium (NBT; Diagnostic Chemicals, Oxford, CT, USA). Slides were coverslipped with Gelvatol mounting medium [12.5% polyvinyl alcohol resin (Air Products and Chemicals, Allentown, PA, USA) and 50% glycerol in PBS]. When NIH 3T3 cells were double-labelled for both DN-erbB4 and AP expression, they were first stained with erbB4 and Cy3 antibodies as described above, fixed again with 4% paraformaldehyde for 10 min, rinsed with PBS, heated to 65 °C for 30 min to inactivate endogenous phosphatase and then stained for AP as described previously.

Morphological analysis of labelled granule cells

All granule cells whose cell body and dendrites could be measured in their entirety were studied. Morphology was analysed using NeuroLucida software (MicroBrightfield, Colchester, VT, USA). The number of dendrites, length of dendrites and area of the cell body were traced and measured. All cells analysed were contained within one or two 50-µm-thick sections.

Results

To test the roles of erbB signalling in postnatal development of cerebellar granule cells, we expressed a DN-erbB4 starting at P1, when granule cell precursors proliferate in the EGL, and then analysed the morphology of neurons once they reached maturity. DN-erbB4 is a truncated human erbB4 protein that completely blocks the activity of all the NRG1 receptors, i.e. erbB2, erbB3 and erbB4 in all cells tested both *in vitro* and *in vivo*, including astrocytes (Rio *et al.*, 1997; Prevot *et al.*, 2003), Schwann cells (Chen *et al.*, 2003; Chen *et al.*, 2006), supporting cells of the inner ear (Stankovic *et al.*, 2004) and neuronal precursors (S.P. Sardi and G. Corfas, unpublished results). Furthermore, DN-erbB4 expression blocks the cellular responses to NRG1 without altering other pathways, including the EGF receptor (erbB1; Prevot *et al.*, 2003) and Notch (Patten *et al.*, 2003). DN-erbB4 was introduced into granule cells in the intact cerebellum by infecting proliferating granule cell precursors at P1 with a retroviral vector. We used the replication incompetent viral vector pLIA, which expresses exogenous genes under the control of the MMLV long-terminal repeat and

has an IRES sequence fused in frame to the human placental AP gene, allowing for visualization of the infected cells (Bao & Cepko, 1997).

To ensure that cells infected with the LIA-DN-erbB4 retrovirus expressed DN-erbB4, we first infected NIH 3T3 cells and stained them with AP histochemistry and with an antibody that recognizes only the human erbB4. Every cell positive for AP also expressed DN-erbB4 (Fig. 1A). To test whether the infection with LIA-DN-erbB4 drove DN-erbB4 expression in cerebellar granule cells *in vivo*, LIA-DN-erbB4 or the control LIA virus were injected into the cerebellar EGL of P1 mouse pups. Animals were allowed to develop and DN-erbB4 expression was analysed by immunostaining at P20, a time at which granule cells have matured. Granule cells expressed DN-erbB4 only in animals injected with LIA-DN-erbB4. Furthermore, all labelled cells were found in the internal granule cell layer (IGL; Fig. 1B), indicating that cells expressing DN-erbB4 were able to migrate from the EGL to the IGL normally.

To determine whether DN-erbB4 expression alters granule cell morphology, we infected granule cell precursors in the EGL at P1 with the control LIA or with LIA-DN-erbB4 retrovirus, harvested tissues at P14 or P20–21 and used AP staining to identify and characterize infected granule cells in the IGL. Infection with either virus resulted in many labelled granule cells in the IGL, confirming that granule cells that were infected in the EGL were capable of migrating to their correct positions in the IGL. AP staining filled the entire granule cell including the cell body, axon, dendrites and their glomeruli, the dendritic terminal protrusions that represent the synaptic contacts of the granule cell dendrites with the mossy fibre and Golgi axon terminals (Fig. 2A). The distinct labelling of the cell morphology with AP allowed us to easily trace the outlines of the cell body, dendrites and synaptic areas (Fig. 2B). Importantly, there appeared to be a similar number of labelled granule cells in LIA-infected and LIA-DN-erbB4-infected cerebella. This suggests that blocking erbB signalling in these cells does not result in cell death and is in agreement with our finding that NRG1 does not promote granule cell survival *in vitro* (Rieff *et al.*, 1999).

We measured the number of dendrites, dendrite length and cell body size for each labelled cell at either P14 ($n = 35$ cells for each virus) or P20–21 ($n = 42$ cells for each virus). Remarkably, only the number of dendrites was affected by DN-erbB4 expression at both ages (see Fig. 3 and Table 1). At P14, LIA-infected granule

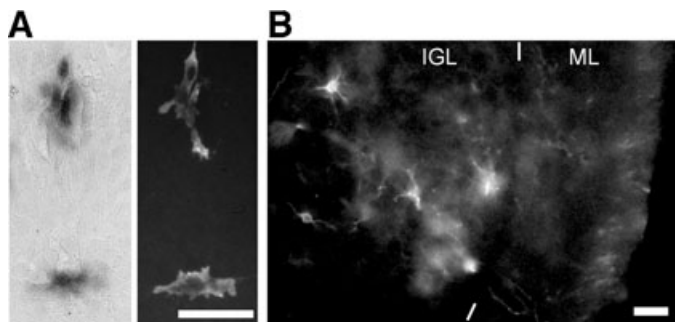


FIG. 1. The DN-erbB4 receptor was expressed in cells infected with LIA-DN-erbB4 *in vitro* and *in vivo*. (A) NIH 3T3 cells infected with LIA-DN-erbB4 were stained for AP activity (left) and with an antihuman erbB4 monoclonal antibody and a Cy3 secondary antibody to detect DN-erbB4 expression (right). All cells that were AP-positive also expressed DN-erbB4. (B) A sagittal section from a P20 mouse cerebellum infected with LIA-DN-erbB4 and stained with an antihuman-erbB4 monoclonal antibody and a Cy3 secondary antibody. Granule cells that express DN-erbB4 are seen in the IGL (A). ML, molecular layer. Scale bars, 100 µm (A), 30 µm (B).

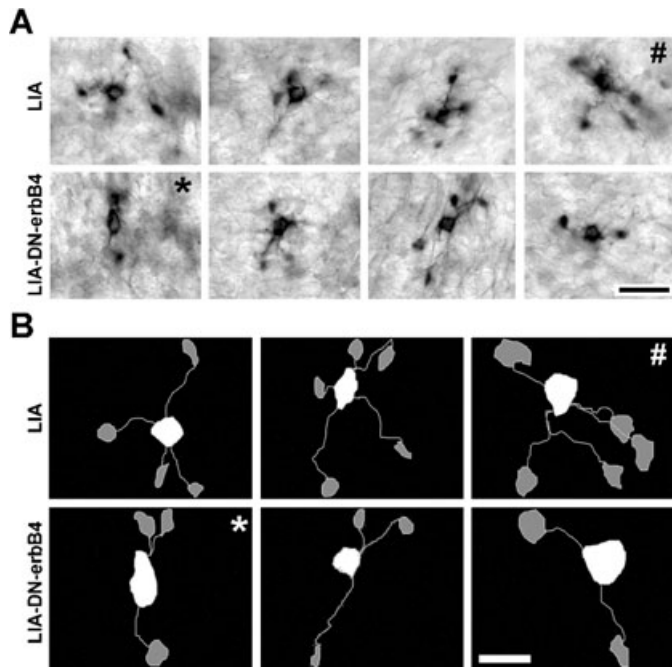


FIG. 2. Examples of granule cells infected with control LIA or LIA-DN-erbB4 virus. (A) Sagittal sections of P20–P21 cerebellum from mice infected with LIA (top panels) or LIA-DN-erbB4 (bottom panels) virus were stained for AP activity. (B) Examples of individual AP-stained granule cells analysed with the NeuroLucida software. The cell bodies (white filling), dendrites (lines) and glomeruli (grey filling) were traced. Traces with * and # correspond to the cells in panel A with the same labels. Scale bars, 20 μm (A), 10 μm (B).

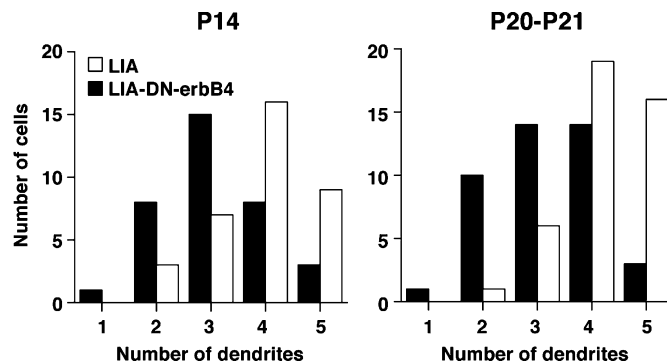


FIG. 3. DN-erbB4 expression reduced the number of dendrites elaborated by granule cells. Histograms of the number dendrites displayed by granule cells infected with LIA (open bars) or LIA-DN-erbB4 (solid bars) virus. Cells were analysed at P14 and P20–21. Cells infected with LIA-DN-erbB4 extended significantly fewer dendrites than cells infected with LIA control virus (P14, $n = 35$ for each virus, $P < 0.001$; P20–21, $n = 42$ for each virus, $P < 0.0001$).

cells elaborated an average of 3.9 ± 0.2 dendrites, while LIA-DN-erbB4-infected cells displayed 3.1 ± 0.2 dendrites ($P < 0.001$; Fig. 3 and Table 1). Similar differences were found at P20–21, when LIA-infected cells had 4.2 ± 0.1 dendrites, while LIA-DN-erbB4-infected cells displayed only 3.2 ± 0.2 dendrites ($P < 0.0001$; Fig. 3 and Table 1). The reduction in dendrite number can be clearly visualized in the histogram, which shows that no LIA-infected cells with just one dendrite were observed, but several cells in this class were found in the DN-erbB4-expressing population (Fig. 3).

TABLE 1. Measurements of granule cell morphology for LIA- or LIA-DN-erbB4-infected mouse granule cells analysed at P14 or P20–21

Age	LIA	LIA-DN-erbB4	<i>P</i> -value
P14			
Number of mice (<i>n</i>)	35	35	
Number of dendrites	3.9 ± 0.2	3.1 ± 0.2	< 0.001
Dendrite length (μm)	18.2 ± 0.9	16.0 ± 1.1	0.14
Area of cell body (μm^2)	65.6 ± 2.2	64.6 ± 2.1	0.75
P20–21			
Number of mice (<i>n</i>)	42	42	
Number of dendrites	4.2 ± 0.1	3.2 ± 0.1	< 0.0001
Dendrite length (μm)	14.7 ± 0.8	14.8 ± 0.7	0.94
Area of cell body (μm^2)	56.1 ± 2.2	58.1 ± 3.2	0.62

Cells from at least five animals were used for each virus and age. The data are presented as average \pm SEM. Statistical significance was determined by unpaired *t*-tests.

Discussion

This study shows that erbB receptor signalling plays an important role in postsynaptic development of granule cells *in vivo* by regulating dendrite formation, and also demonstrates that NRG1-erbB signalling plays diverse roles in granule cell development. Previously we showed that, during their migration, granule cells express NRG1 but not erbB receptors, and that they use the ligand to regulate radial glia morphology (Rio *et al.*, 1997). Based on those results we proposed that, during granule cell migration, erbB receptor signalling is necessary in Bergmann glia, not in the neurons. The results presented here, showing that granule cells expressing DN-erbB4 migrate normally, indicates that this hypothesis was correct. After these neurons arrive at their final destination in the internal granule cell layer, they stop expressing NRG1 and begin to express erbB4, which localizes to their postsynaptic terminals (Rieff *et al.*, 1999). The present results together with our previous *in vitro* studies (Rieff *et al.*, 1999) show that, after granule cell migration is complete, erbB signalling in granule cells becomes critical for their further development. Thus NRG1-erbB signalling plays dynamic roles in the development of cerebellar granule cells.

One of the earliest biological activities attributed to NRG1 was the induction of expression of ACh receptors in skeletal muscle, an observation that led to the hypothesis that this factor could be important for the formation of the postsynaptic apparatus at the neuromuscular junction (Falls *et al.*, 1993; Fischbach & Rosen, 1997). Studies of the pattern of expression of NRG1 suggested that this signalling pathway could also be important in the regulation of neurotransmitter expression in neurons (Corfas *et al.*, 1995). Indeed, NRG1-erbB signalling has been shown to regulate the expression of several neurotransmitter receptors in the CNS, including ACh (Liu *et al.*, 2001), GABA_A (Rieff *et al.*, 1999; Okada & Corfas, 2004; Xie *et al.*, 2004) and NMDA (Ozaki *et al.*, 1997; Stefansson *et al.*, 2002; Gu *et al.*, 2005) receptors. We now demonstrate that erbB signalling plays a key role in the morphological development of dendrites in the most abundant neuronal type in the CNS. As NRG1 has been shown to induce neurite outgrowth in other cell types (Bermingham-McDonogh *et al.*, 1996; Vaskovsky *et al.*, 2000; Gerecke *et al.*, 2004), it is possible that erbB signalling may play a role in dendrite formation in other neuronal populations.

Even though NRG1-erbB signalling regulates aspects of astrocyte development and function (Pinkas-Kramarski *et al.*, 1994; Prevot *et al.*, 2003), and these cells regulate synapse formation (Ullian *et al.*, 2004; Christopherson *et al.*, 2005), our experimental approach, *i.e.* the

direct inhibition of erbB signalling in single neurons within the intact brain, indicates that the effects of erbB signalling on dendritic development occur in the neurons in a cell-autonomous fashion. As the number of dendrites but not their length is altered by the loss of erbB signalling, and this defect is observed by P14, before granule cell maturation is complete, we conclude that this pathway regulates the initial formation of dendrites. Furthermore, by P20–21, a time at which granule cells are considered mature, the DN-erbB4-expressing cells still display fewer dendrites, showing that the reduction in dendrite number is not a reflection of delayed dendritogenesis but rather a true developmental defect in dendrite formation.

It is important to note that DN-erbB4 expression did not block the formation of all dendrites nor did it affect the length of the remaining dendrites. Furthermore, each dendrite formed in the absence of erbB signalling had a glomerulus (the large and complex synaptic terminal where granule cell dendrites receive the inputs from the mossy fibre afferents and Golgi axons) which appeared normal under visual examination. Thus, once a granule cell dendritic process is extended, its maturation, including the formation of the synaptic terminal, appears to be independent of erbB signalling. However, whether those terminals are functionally normal remains to be determined.

Our results suggest that other signals, in addition to NRG1, may also contribute to granule cell dendritic growth *in vivo*. Dendritic morphology has been shown to be influenced by multiple environmental cues, including guidance molecules, the neurotrophins and neuronal activity (McAllister *et al.*, 1999; Scott & Luo, 2001; Whitford *et al.*, 2002; Van Aelst & Cline, 2004). In granule cells, the neurotrophins may play a role in dendritic development as brain-derived neurotrophic factor enhances neurite outgrowth and neurotrophin 3 (NT3) increases neurite fasciculation and branching of cerebellar granule cells in culture (Segal *et al.*, 1995). Thus, neurotrophin signalling may also contribute to granule cell dendritic development *in vivo*.

Most studies on mammalian dendritic development have been performed in tissue cultures (McAllister *et al.*, 1999; Scott & Luo, 2001; Whitford *et al.*, 2002; Van Aelst & Cline, 2004). We now provide insights into the mechanisms of dendrite formation *in vivo* and identify a signalling pathway that appears to orchestrate a general program of postsynaptic development, from the initiation of dendritic formation to the formation and maturation of the postsynaptic apparatus. It would be interesting to investigate whether erbB signalling regulates dendritic morphogenesis in other neuronal populations, in particular in the hippocampus and prefrontal cortex, where NRG1-erbB signalling has been shown to regulate other aspects of postsynaptic development such as the expression of ACh (Liu *et al.*, 2001), GABA_A (Okada & Corfas, 2004) and NMDA (Stefansson *et al.*, 2002; Gu *et al.*, 2005) receptors. This is of particular importance as NRG1 has been recently linked to schizophrenia (Corfas *et al.*, 2004), a disease in which defects in dendritic development have been demonstrated (Lewis *et al.*, 2003).

Because expression of DN-erbB4 blocks activation of erbB2, erbB3 and erbB4 by all their cognate ligands, the specific ligands and receptors involved in granule cell dendrite development remain to be determined. Our earlier *in vitro* study indicates that type I NRG1, which is expressed by neurons that form the mossy fibers (Corfas *et al.*, 1995), induces at least some aspects of granule cell maturation. Neuronal activity has been implicated in dendritic development, and the possibility that the effects of erbB signalling on dendrite formation are secondary to changes in neurotransmission need to be considered. However, it is unlikely that the effects of erbB signalling on neurotransmitter receptor expression (Rieff *et al.*, 1999) precede those on the initiation of dendrite outgrowth. A more appealing possibility is

that erbB receptor signalling regulates the activity of small GTPases (Van Aelst & Cline, 2004) of CamKII-NeuroD signalling (Gaudilliere *et al.*, 2004), which have been clearly implicated in dendrite development. Further studies on the mechanism of erbB receptor signalling could help to elucidate the mechanisms underlying dendritic development.

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Abbreviations

AP, alkaline phosphatase; Cy3, cyanine 3; DN-erbB4, dominant negative erbB4 receptor; EGL, external granule cell layer; GABA_A, γ -aminobutyric acid-A; IGL, internal granule cell layer; NMDA, *N*-methyl D-aspartate; NRG1, neuregulin 1; P, postnatal day; PBS, phosphate-buffered saline.

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