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Neuregulin and erbB Receptors Play a Critical Role in Neuronal Migration

Carlos Rio,*‡ Heather I Rieff,*†‡ Peimin Qi,* and Gabriel Corfas*†

*Division of Neuroscience and Department of Neurology
Children's Hospital and Harvard Medical School
300 Longwood Avenue
†Program in Neuroscience
Harvard Medical School
220 Longwood Avenue
Boston, Massachusetts 02115

Summary

The migration of neuronal precursors along radial glial fibers is a critical step in the formation of the nervous system. In this report, we show that neuregulin–erbB receptor signaling plays a crucial role in the migration of cerebellar granule cells along radial glial fibers. Granule cells express neuregulin (NRG), and radial glia cells express erbB4 in the developing cerebellum and in vitro. When the glial erbB receptors are blocked, neurons fail to induce radial glia formation, and their migration along radial glial fibers is impaired. Moreover, soluble NRG is as effective as neuron–glia contact in the induction of radial glia formation. These results suggest that the activation of glial erbB4 by NRG is an early critical step in the neuronal migration program.

Introduction

Neuronal migration is a critical step in the construction of a functional nervous system, and its failure has been implicated in several disease states such as epilepsy, mental retardation, and chronic neurological disability in childhood (Aicardi, 1994). Most immature neurons migrate from the germinal layers to their final destination along radial glial fibers, which provide them with a scaffold for their organized movement. This migration process is a complex developmental program involving a series of steps, including cell–cell recognition, cell–cell adhesion, cell motility, and finally detachment from the glial fibers once neurons reach their destination (Rakic et al., 1994). As in other developmental processes, many molecules must participate in neuronal migration. Some molecules, such as cell adhesion molecules and motor proteins, may contribute to discrete steps in the migration act; others, like extracellular signaling molecules, may regulate the activation and/or termination of the migration program.

The molecular mechanisms that underlie neuronal migration have been the subject of intense investigation. A useful model system to study neuronal migration is the movement of cerebellar granule cells along Bergmann glial fibers. Cerebellar granule cells proliferate in the external germinal layer (EGL) and migrate inward along Bergmann radial fibers to their final destination, the internal granule cell layer (IGL) (Rakic, 1971). In rodents, this migration takes place during the first 2 postnatal weeks. Granule cell migration has been studied in vitro in both cerebellar slices and in cocultures of dissociated cerebellar granule cells and astroglia. These studies have implicated a number of molecules in steps of the migration process, for example, cell adhesion molecules, i.e., AMOG (Antonicek et al., 1987; Gloor et al. 1990) and astrotactin (Zheng et al., 1996); ion channels, i.e., NMDA receptors (Komuro and Rakic, 1993) and Ca^{2+} channels (Komuro and Rakic, 1992). In addition, studies of mutant mouse lines with defects in brain development have led to the identification of other molecules, including potassium channels in the case of the *weaver* mutant (Rakic and Sidman, 1973; Patil et al., 1995); the extracellular matrix protein reelin in the *reeler* mutant (Caviness and Sidman, 1973; D'Arcangelo et al., 1995; Hirotsune et al., 1995); and p35, an activator of Cdk5 (Chae et al., 1997). However, until now, no extracellular signals that regulate the activation of the migration program have been identified.

Growth and differentiation factors are known to regulate complex developmental processes and are likely to play an important role in neuronal migration. One of these factors, neuregulin (NRG), may play a key role in the activation and/or control of the migration program in the cerebellum. NRG is a member of the epidermal growth factor (EGF) family of growth factors. It was identified in several laboratories based on different biological activities, including induction of proliferation of Schwann cells (GGF; Marchionni et al., 1993), induction of acetylcholine receptor in muscle (ARIA; Falls et al., 1993), and activation of the tyrosine kinase receptor erbB2 (Neu Differentiation Factor [Peles et al., 1992] and Heregulin [Holmes et al., 1992]). Alternative splicing of the NRG gene leads to the expression of several different isoforms of NRG. Some of the isoforms are expressed as a transmembrane precursor while others are produced as a secreted protein. All NRG isoforms contain in their extracellular portion an EGF-like domain that appears to be responsible for NRG's biological activities (Carraway and Burden, 1995; Fischbach and Rosen, 1997). NRG activates the tyrosine kinase receptors erbB2, erbB3, and erbB4, which are members of the EGF receptor family (Carraway and Burden, 1995). erbB3 and erbB4, but not erbB2, bind NRG. However, upon ligand binding, erbB3 and erbB4 receptors can form homo- or heterodimers, which can include erbB2. Dimerization leads to the activation of their tyrosine kinase domains and the initiation of intracellular signaling events (reviewed by Carraway and Cantley, 1994).

The nervous system is one of the sites of highest NRG expression (Holmes et al., 1992) where it is mostly expressed by neurons (Chen et al., 1994; Corfas et al., 1995). In the peripheral nervous system, NRG has been shown to mediate neuron–glia interactions, where neuronally produced NRG induces the proliferation or survival of Schwann cells (Morrissey et al., 1995; Syroid

‡These authors contributed equally to this work.

et al., 1996; Trachtenberg and Thompson, 1996). Other studies suggested that NRG may play a role in neuron–glia interactions in the central nervous system as well (Pinkas-Kramarski et al., 1994; Vartanian et al., 1994; Canoll et al., 1996). In the cerebellum, NRG mRNA is expressed by migrating granule cells, and NRG expression in the cerebellum is mostly restricted to the period of neuronal migration (Corfas et al., 1995). Based on these precedents, we studied the role of NRG in the interactions between granule cells and cerebellar glia in the developing cerebellum. We demonstrate here that during the period of cerebellar granule cell migration, NRG is expressed by cerebellar granule cells while erbB4 is expressed by the Bergmann glia. We also show that NRG–erbB receptor signaling in glia is necessary for several steps in the neuronal migration program: NRG–erbB receptor signaling is necessary and sufficient for the neuronal induction of radial glia morphology and is also critical for the migration of granule cells along glial fibers. These results suggest that NRG and its receptors play a critical role in neuron–glia interactions and neuronal migration in the developing cerebellum.

Results

Expression of NRG and erbB4 in the Developing Cerebellum

We first analyzed the pattern of expression of NRG and erbB receptors in the developing cerebellum. During the period of granule cell migration, NRG is expressed by these neurons, and the expression is evident at every step of their migration. At P0, when most granule cells are in the EGL, all neurons in the EGL are labeled, including the proliferating and the premigratory cells (Figure 1A). At P6, when migration is at its peak, labeled granule cells can be found in the EGL, the IGL, and while migrating through the developing molecular layer (Figure 1A). By P12, most of the labeled cells are in the IGL. By P19, NRG immunoreactivity is restricted to the IGL and is mostly absent from the superficial layer of the cerebellum. Examination at high magnification showed that NRG immunoreactivity is concentrated in the cell body of the premigratory and migratory cells (Figure 1C). These results were identical using anti-NRG antibodies directed either to the extracellular domain or to the intracellular domain, suggesting that NRG may be expressed as a transmembrane protein in granule cells. The expression of NRG in the developing cerebellum was confirmed by western blot (Figure 2C). In the adult, NRG expression in the IGL decreases, and NRG immunoreactivity is mostly found in the molecular layer of the cerebellum, where it appears to be concentrated at synaptic terminals (Sandrock et al., 1995). The same pattern of expression was observed by in situ hybridization (Corfas et al., 1995).

We also analyzed the expression of the erbB receptors by immunohistochemistry, Western blot, and in situ hybridization. We found that, of all the NRG-receptors, erbB4 is most abundantly expressed in the developing cerebellum. Double labeling with erbB4 and GFAP antibodies showed that erbB4 is expressed only in the Bergmann glia cells, where it is mostly concentrated in the glial fibers, with staining in the cell bodies being much weaker (Figure 1D). Radial glial fibers express erbB4 precisely during the period of granule cell migration. erbB4 expression in the radial glia was high from P0 to P12, but it was practically absent by P19, after migration is complete (Figure 1B). As with NRG, the pattern of expression of erbB4 in the adult changes; the receptor is expressed mostly by mature granule cells (not shown). The expression of erbB4 in the developing cerebellum was confirmed by Western blot (Figure 2F). We could not detect erbB2 or erbB3 expression in the Bergmann glia by immunohistochemistry and in situ hybridization (not shown).

The expression of NRG by migrating granule cells and erbB4 by the radial glial fibers suggested to us that this ligand–receptor pair may mediate interactions between these cells during neuronal migration.

Expression of NRG and erbB4 in the Cerebellar Cells in Culture

To study possible roles of NRG as an inducer of the neuronal migration program, we adapted a previously developed in vitro system to study granule cell–astroglia interactions and neuronal migration (Hatten, 1985; Edmondson and Hatten, 1987).

First it was necessary to determine whether the expression of NRG and erbB4 was appropriately maintained when the cerebellar cells are placed in culture. We generated pure cultures of cerebellar granule cells and astroglia using Percoll gradients and panning as described by Hatten (1985). In these cultures, we found that, as in vivo, purified granule cells express NRG (Figures 2A–2C), and purified astroglia express erbB4 (Figures 2D and 2F). NRG immunoreactivity in granule cells was localized to the neurites and cell body (Figures 2A and 2B). As granule cells mature in culture, NRG is down-regulated (not shown); this parallels the down-regulation of NRG expression in granule cells in vivo. erbB4 expression by the glial cells was maintained in vitro for at least three passages. Astroglial cells respond to acute application of NRG with the induction of tyrosine phosphorylation of p185 (Figure 3A) and the phosphorylation of the transcription factor CREB in serine 133 (Figure 3B). These results indicate that, for a few days in vitro, cerebellar cells maintain the same pattern of NRG and erbB4 expression seen in the developing cerebellum, allowing us to test whether NRG signaling mediates some of the neuronal effects on the glial cells in vitro.

Generation and Characterization of a Dominant-Negative erbB4 Receptor

To test whether NRG is the mediator of neuron–glia interactions, it was necessary to block the function of neuronal NRG. For this purpose, we developed a dominant-negative erbB4 receptor (DN-erbB4) lacking most of the intracellular domain, including the tyrosine kinase domain and the tyrosine phosphorylation sites. Transfection of this plasmid into COS cells resulted in the expression of a protein of the expected molecular weight (130 kDa) (Figure 4A). Upon NRG binding, erbB receptors form homo- and heterodimers and become active. We therefore hypothesized that expression of DN-erbB4 should block the activation of all NRG receptors, i.e.,

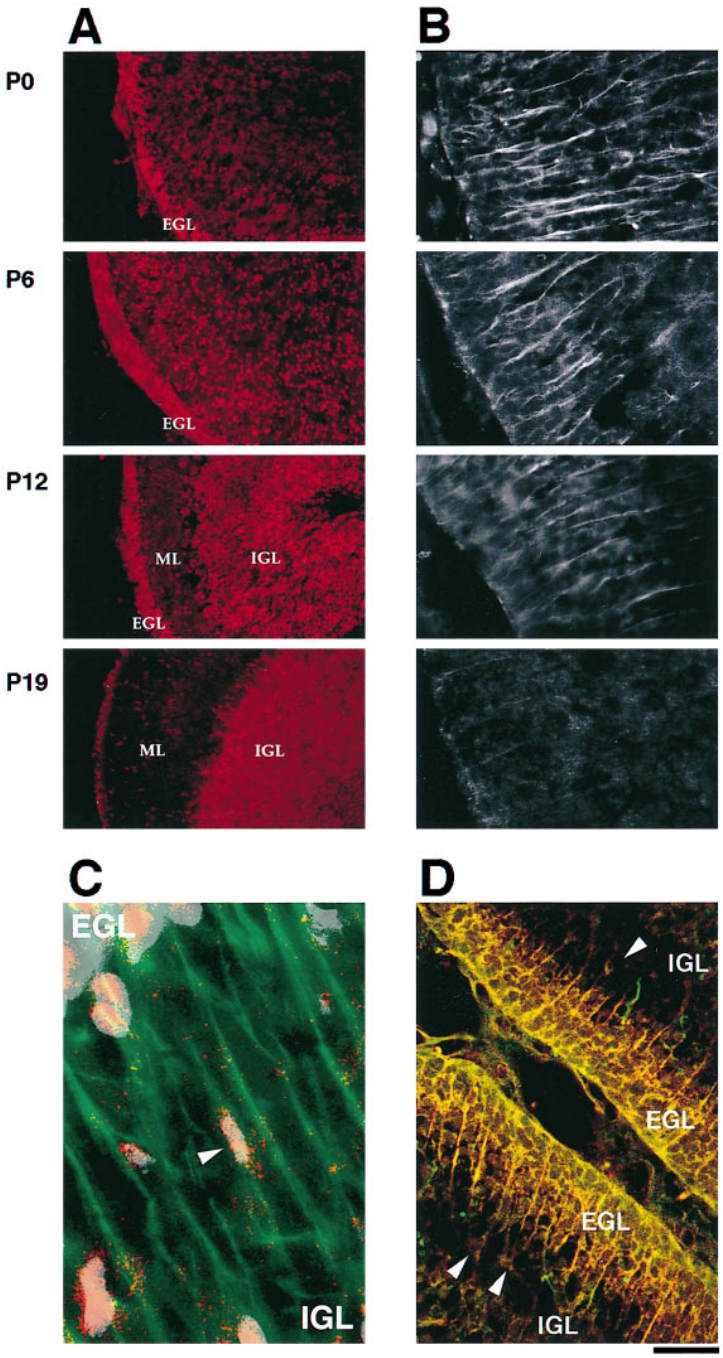

Figure 1. Expression of NRG and erbB4 in the Developing Cerebellum

(A) Sagittal sections of postnatal rat cerebellum stained with a NRG antibody (HM94) and a Cy3-conjugated secondary antibody. NRG immunoreactivity colocalizes with granule cells in different stages of migration. By P19, when granule cell migration is complete, the NRG staining is nearly absent in the external cerebellar layers. The scale bar, 100 μm.

(B) Confocal images of sagittal sections of postnatal rat cerebellum stained with the erbB4 antibody (0615) and a Cy3-conjugated secondary antibody. Bergmann glial fibers at P0 and P6 have high erbB4 immunoreactivity. By P19, the staining is almost absent, paralleling the decrease in NRG expression in the granule cells. The scale bar, 30 μm.

(C) NRG staining of a granule cell migrating through the developing molecular layer (arrowhead). A section through a P12 rat cerebellum was stained with an anti-GFAP MAb (FITC), which labels radial glial fibers, and a NRG polyclonal antibody, HM209 (Cy3). Hoechst dye (shown here in gray) was used to label nuclei. Pictures were obtained in a confocal microscope at different focal planes in order to give a clear image of the relative localization of the different stains. The superimposition of the images shows that NRG staining extends beyond the granule cell nucleus. The scale bar, 20 μm.

(D) Confocal image of P6 rat cerebellum double labeled with an anti-GFAP MAb (FITC) and an erbB4 polyclonal antibody, 0615 (Cy3). The yellow staining shows the colocalization of GFAP and erbB4 in the radial glial fibers. Arrowheads point to the Bergmann glia cell bodies, which are more weakly stained compared to the glial fibers. The scale bar, 50 μm.

erbB2, erbB3, and erbB4. To test this hypothesis, we used the L6 and C2C12 muscle cell lines, which mostly express erbB2 and erbB3 receptors (Corfas, unpublished data). We established stable muscle cell lines expressing DN-erbB4 and confirmed by immunofluorescence that the protein was expressed. The immunoreactivity was localized to the cell surface, suggesting that it was expressed as a transmembrane protein (not shown, but see Figure 4C). When these cells were treated with soluble NRG, we found that expression of DN-erbB4 blocked the induction of tyrosine phosphorylation of p185 (Figure 4B). These results demonstrate that expression of DN-erbB4 can block the NRG-induced erbB receptor signaling in transfected cells. We could then use DN-erbB4 to test the role of NRG in the interactions between granule cells and astroglia.

As a first step, we tested whether DN-erbB4 could be efficiently expressed in primary cultures of cerebellar astroglia. We transfected primary cerebellar astroglial cells with the plasmid coding for DN-erbB4 or the Green Fluorescent Protein (GFP). By immunostaining with an antibody directed to the extracellular domain of human-erbB4, we found that ~30% of the glial cells expressed DN-erbB4 (not shown, but see Figure 4C). The same percentage of transfected cells was obtained using the plasmid expressing GFP (not shown). As in the muscle

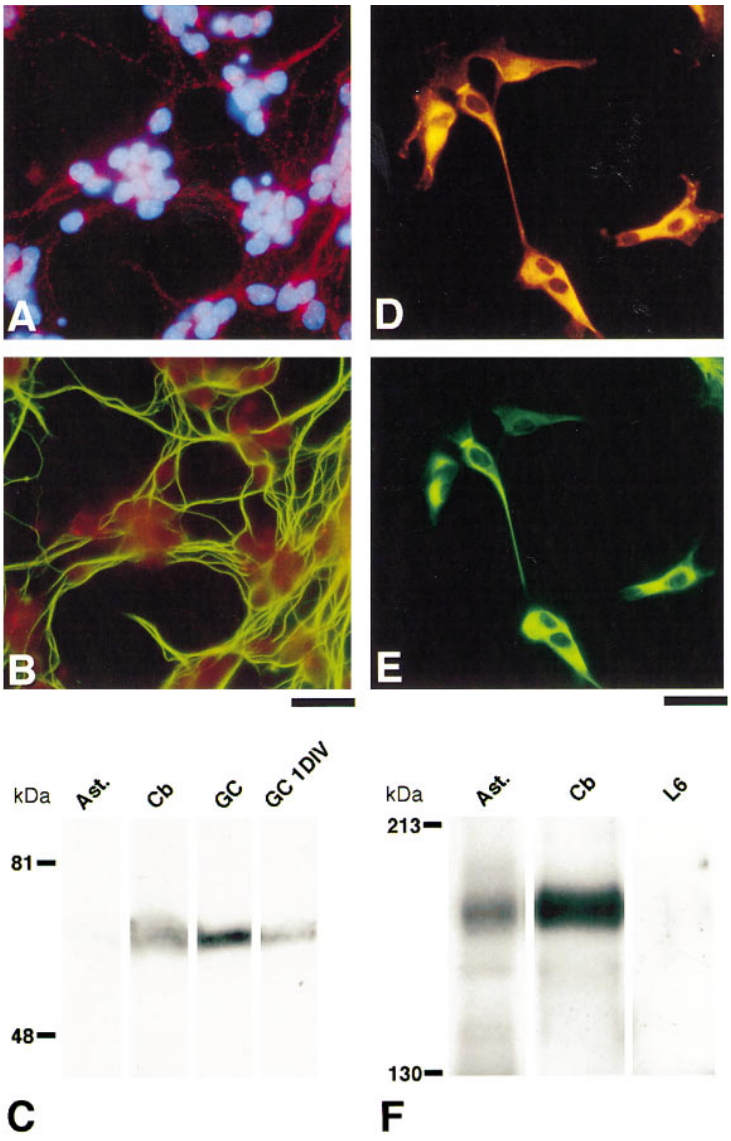

Figure 2. Expression of NRG and erbB4 Cerebellar Cells in Culture

(A and B) Purified cerebellar granule cells (1 DIV) were stained with an anti-NRG antibody, 1915 (Cy3); a monoclonal granule cell-specific antibody, Q600 (FITC); and Hoechst dye to label the nuclei (blue). The same field is shown in both panels. NRG immunoreactivity is present in the neuronal processes (A) and in the cell bodies of the granule cells (B). The scale bar, 30 μm.

(C) Western blot analysis of NRG expression in cerebellar cells with antibody 1915. Lysates of the different tissues were resolved by 10% SDS–PAGE. Ast, astroglial cells; Cb, P6 rat cerebellum; GC, cerebellar granule cells 5 hr after dissociation; and GC 1DIV, granule cells 1 day in vitro. The immunoreactive band has a molecular weight of ~65 kDa.

(D and E) Purified cerebellar astroglia express erbB4. Astroglial cells were double labeled with an erbB4 polyclonal antibody (0615) and a Cy3-conjugated anti-rabbit secondary antibody (D), and with a GFAP MAb and FITC-conjugated anti-mouse secondary antibody (E). The scale bar, 30 μm.

(F) Western blot analysis of erbB4 expression in cerebellar cells. Plasma membrane proteins were isolated from cerebellar astroglia (Ast), P6 rat cerebellum (Cb), and L6 muscle cells (L6). Equal amount of protein was loaded in each lane, resolved by 5% SDS–PAGE, and detected using the polyclonal antibody anti-erbB4 (C18).

cells, DN-erbB4 immunoreactivity was on the cell surface, suggesting that it is expressed as a transmembrane protein. Finally, we tested whether expression of DN-erbB4 blocked the effects of NRG on CREB phosphorylation in DN-erbB4-expressing glia. As shown in Figure 4C, expression of DN-erbB4 in cerebellar astroglia blocked the induction CREB phosphorylation by NRG but not by serum. While serum induced P-CREB in the nuclei of 95% of the DN-erbB4-expressing cells, only 3% of glia expressing DN-erbB4 contained P-CREB immunoreactivity after exposure to NRG, the same level found in unstimulated cells. These results demonstrate that expression of DN-erbB4 blocks glial responses to NRG in a specific manner.

NRG Is Critical for the Neuronal Induction of Radial Glia Morphology

Since granule cells cause astroglia to adopt a radial morphology (Hatten, 1985), we tested the ability of DN-erbB4 to block this neuronal effect. Cerebellar astroglial cells were transfected with DN-erbB4 or with a control plasmid (GFP) and kept in culture for 24 hr to allow expression of the transgenes. The cells were then stimulated by the addition of freshly dissociated granule cells (5–10 neurons per glia) and incubated for another 24 hr. Cells were then fixed and stained with anti-human-erbB4 antibodies to detect the expression of DN-erbB4, as well as with anti-GFAP antibodies to visualize the glial shapes. When we looked at individual glial cells in direct contact with neurons, we found that the neurons failed to induce radial glial morphology in cells expressing DN-erbB4. However, in the same cultures, cells not expressing DN-erbB4 responded normally to neuronal contact (Figures 5 and 6). Moreover, transfection of glia with DN-erbB4 or the control plasmid had no effect on the morphology of unstimulated cells. The percentage of glia in contact with neurons, and the number of neurons per glial cell was the same for DN-erbB4-expressing cells and control cells, suggesting that cell adhesion was not affected (not shown). These results indicate

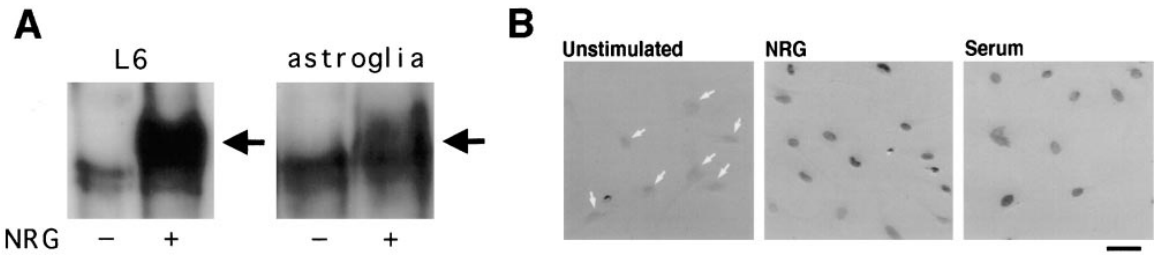

Figure 3. Astroglia in Culture Respond to NRG Stimulation

(A) NRG induces p185 tyrosine phosphorylation in astroglia cells. Confluent cultures of L6 or astroglia were stimulated with 1 nM NRG (+) or vehicle (−) for 5 min. Lysates were resolved by 5% SDS–PAGE, and the blot was probed with an antiphosphotyrosine antibody (4G10). The arrow shows the 185 kDa phosphorylated protein.

(B) NRG induces CREB phosphorylation in astroglial cells. Astroglial cells in serum-free medium were treated for 30 min with vehicle (unstimulated), NRG (2.25 nM), or horse serum (final concentration 5%). Cells were fixed, incubated with antibodies specific for CREB phosphorylated in serine 133, and stained by peroxidase reaction. White arrows point out the nuclei of the unstimulated cells. The scale bar, 35 μm.

that erbB receptor signaling in the glia is critical for the neuronal induction of radial glial morphology.

We then tested whether NRG alone could mimic the effects of neurons on glial morphology. Astroglia were stimulated for 24 hr with soluble EGF-like domain of NRG (1 nM), and the morphology of the glia was analyzed after fixation. This treatment caused a dramatic morphological change in the glia similar to that induced by neuronal contact (Figure 5). The percentage of cells with radial morphology was the same whether the glia were treated with NRG or were in contact with neurons (Figure 6). The effects of NRG on glia morphology were completely blocked by expression of DN-erbB4 in the glia (Figure 6). These results show that NRG produced by granule cells is responsible for the morphological change in the glia, which is the initial event leading to neuronal migration. Moreover, they demonstrate that NRG is necessary and sufficient for the induction of glial morphological change.

DN-erbB4 Expression Impairs the Migration of Granule Cells along Glial Fibers

The results described above show that NRG–erbB receptor signaling is important for the early neuron–glia interactions that lead to neuronal migration. We wanted to test whether these signaling events are also important for later stages in the migration program. Since granule cell migration in vitro only occurs along the radial glial

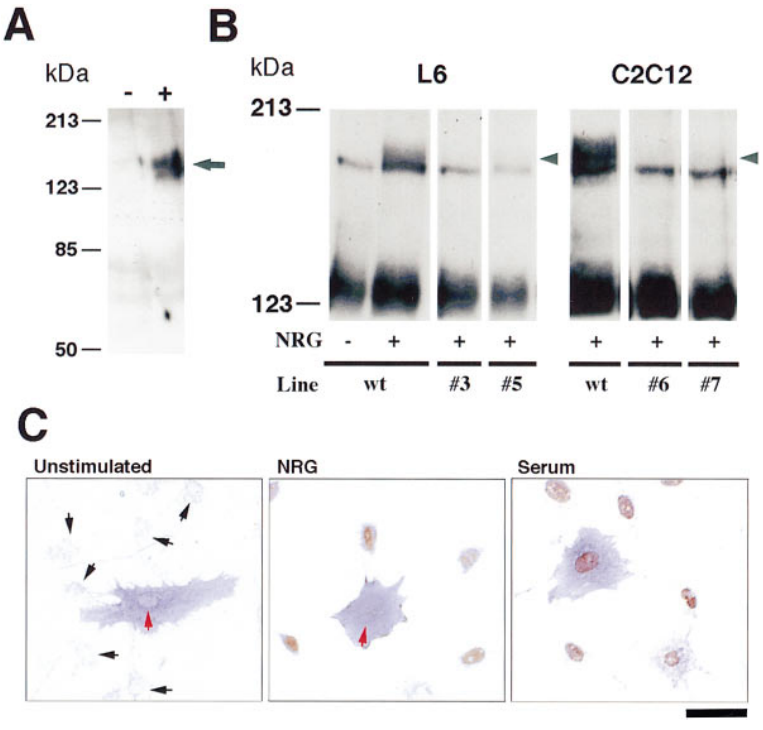

Figure 4. The Dominant-Negative erbB4 Receptor Blocks NRG Function

(A) Flag Western blot of COS7 cells, untransfected (−), or transfected with the DN-erbB4 plasmid (+). A protein containing the flag epitope is seen only in the transfected cells. The protein has the molecular weight expected for erbB4, lacking most of the intracellular domain, i.e., ~130 kDa (arrow).

(B) Phosphotyrosine immunoblot of L6 and C2C12 muscle cell lines. Wild-type muscle cell lines and lines expressing DN-erbB4 were treated for 5 min with NRG (1 nM). Lysates were resolved by 5% SDS–PAGE. In wild-type cells, NRG induces tyrosine phosphorylation of the erbB receptors (p185). Expression of DN-erbB4 in different stably transfected clones of L6 (#3 and #5) and of C2C12 (#6 and #7) results in the absence of NRG-induced p185 phosphorylation (arrowheads).

(C) Expression of DN-erbB4 in primary cultures of cerebellar astroglia blocks NRG-induced CREB phosphorylation. Glial cells transfected with the DN-erbB4 plasmid were kept in serum-free media for 12 hr. Cells were then treated for 30 min with vehicle (unstimulated), NRG (2.25 nM), or horse serum (final concentration 5%). Cells were fixed, and incubated with antibodies specific for CREB phosphorylated in serine 133 and anti-human erbB4 (H4.77.16). The phosphorylated CREB was visualized by the DAB-peroxidase reaction (brown nuclei), and the erbB4-expressing cells by the alkaline phosphatase-NBT/BCIP reaction (blue cells). Red arrows point out the nuclei of the human erbB4-expressing cells (in blue), and the black arrows indicate unlabeled cells. The scale bar, 25 μm.

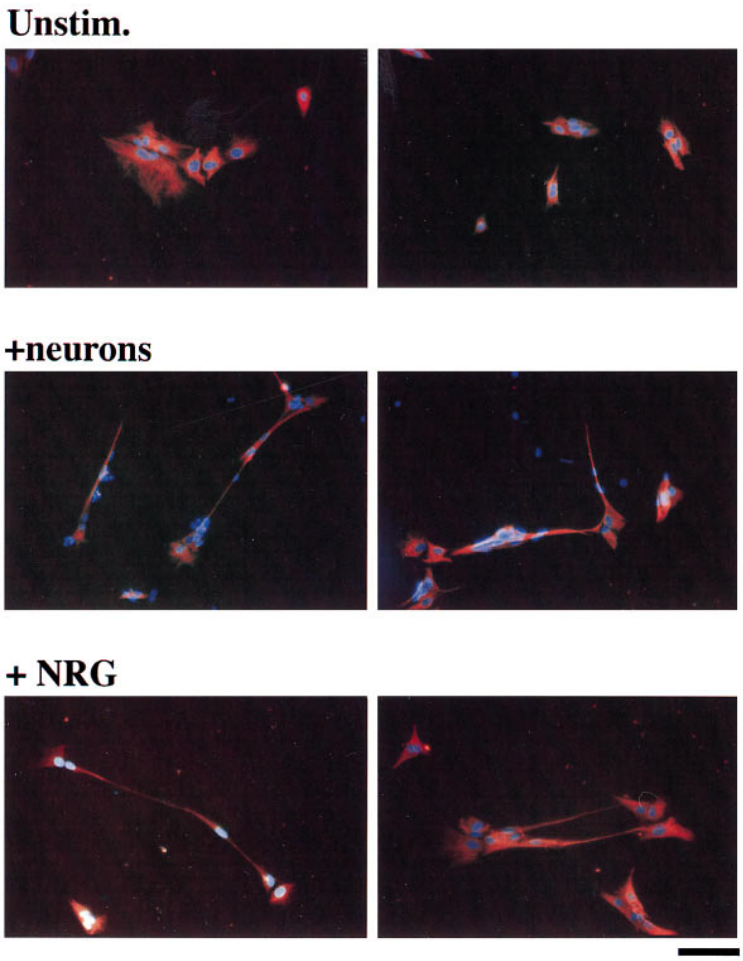

Figure 5. NRG and Granule Cells Induce Radial Morphology in Cerebellar Astroglia

Glial cells in culture were left unstimulated (top sections), cocultured for 24 hr with granule cells (middle sections), or treated for 24 hr with 1 nM NRG (bottom sections). Cells were fixed and stained with a GFAP polyclonal antibody and a Cy3-conjugated donkey anti-rabbit secondary antibody. Cell nuclei were labeled with the dye Hoechst 33342. While unstimulated cells have a fibroblastic appearance, the cells in contact with neurons (see small Hoechst-labeled nuclei) or treated with NRG had a radial morphology with very long processes. The scale bar, 70 μm.

processes (Edmondson and Hatten, 1987), we needed to monitor the movements of granule cells along the radial processes of DN-erbB4-expressing glia. Even though expression of DN-erbB4 resulted in a reduction in the number of radial glia cells, a certain percentage of unstimulated glia already possess radial processes, both in the control group as well as cells expressing DN-erbB4 (Figure 6). This basal level of glial morphological differentiation allowed us to test the importance of erbB receptor signaling in the movement of neurons along radial glial processes.

Glial cells were transfected with the plasmids expressing DN-erbB4 and GFP. Under the conditions used, we found that all glial cells expressing GFP also expressed DN-erbB4. As controls, we used untransfected glia or glia transfected with pcDNA3 and GFP plasmids. Twenty-four hours later, freshly dissociated granule cells were added, and the cells were cocultured overnight. Then, radial glial cells that expressed GFP and had neurons positioned on their radial processes were identified, and the movement of the granule cells along these radial processes was monitored by time-lapse microscopy (Figure 7). When the behavior of granule cells on glia expressing DN-erbB4 was compared to the control cells, we found that expression of DN-erbB4 led to a dramatic effect on neuronal migration. The behavior of neurons over control glia, both untransfected and transfected with the empty vector, was identical to that described by others (Edmondson and Hatten, 1987). Most cells moved along the radial fibers, and the average speed of migration was 27.5 ± 1.8 μm/hr (n = 49) (Figures 7A and 7C). However, most neurons attached to radial processes of DN-erbB4-expressing glia failed to move significantly (>10 μm/hr) during the time of examination. The average speed of migration for these cells was 15.9 ± 2.0 μm/hr (n = 31) (Figures 7B and 7C). The differences between the speed of migration were highly significant ($P < 0.0001$). All neurons located along radial glia expressing DN-erbB4 moved at least a few microns during the period of recording, suggesting that blocking erbB receptor signaling in glia does not result in a complete immobilization of the neurons, but rather in a reduction of neuronal migration.

Discussion

Our results demonstrate that NRG–erbB receptor signaling is critical for the neuron–glia interactions that lead to neuronal migration. This represents the first example of a ligand–receptor pair required for the complex process of neuronal migration.

We have shown that NRG and erbB receptors play a

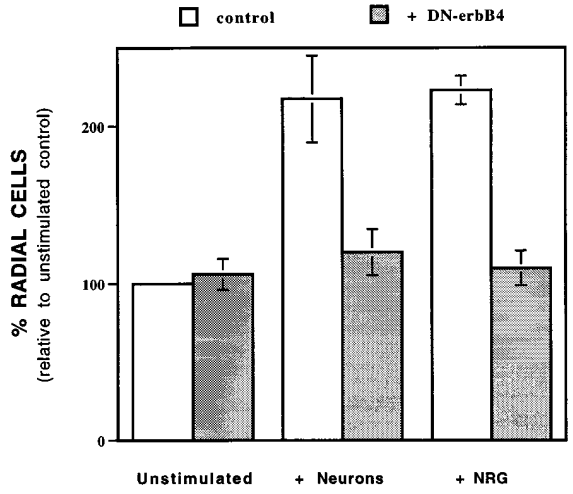

Figure 6. DN-erbB4 Blocks the Effects of Neurons and NRG on Glial Morphology

The histogram depicts the effects of granule cells and NRG on the morphology of control glial cells and glial cells expressing DN-erbB4. Cerebellar astroglia were transfected with the GFP plasmid (control) or the DN-erbB4 plasmid, and incubated for 24 hr. Then, the glia were either left unstimulated, or stimulated for 24 hr by coculturing with granule cells (5–10 neurons/glia) or by adding 1 nM NRG to the medium. Cells were fixed, stained, and their morphology was analyzed (see Experimental Procedures). Neurons and NRG induce radial morphology of control cells but not of glia that express DN-erbB4. The results represent the mean ± SEM of three independent experiments. The percentage of radial cells in control glial cultures treated with NRG or neurons differs significantly from the other groups ($P < 0.0031$ by ANOVA).

crucial role in at least two steps of the migration program. In the early stages of the migration program, NRG–erbB receptor signaling is required for the neuronal induction of radial glia morphology; in the later stages, erbB receptor signaling in the glia appears to be essential for the movement of the granule cells along the radial glial fibers. The latter was demonstrated by showing that most neurons positioned on glial fibers, which would normally migrate, failed to move when the radial glia express DN-erbB4. These results indicate that NRG–erbB receptor signaling is also important for a step in neuronal migration that occurs subsequent to the induction of radial glia morphology. Moreover, these findings demonstrate the important contribution of glia to the migration process. It is not sufficient for a glia to be radial to support migration, but other more subtle differentiation events must underlie the ability of glial fibers to promote neuronal movement.

The pattern of expression of NRG and erbB4 in the developing cerebellum indicate that these molecules may play a role in neuronal migration in vivo. In the developing mouse cerebellum, morphologically distinct Bergmann glia appear at E15 (Del Cerro and Swarz, 1976), 2 days later than the arrival of the granule cells' precursors to the surface of the developing cerebellum at E13 (Hatten et al., 1982). Moreover, we have observed NRG mRNA in the superficial layers of the embryonic (E14) cerebellum (Corfas, unpublished data). Therefore, it is possible that granule cell–derived NRG may be involved in the initial formation of Bergmann glial fibers in the developing cerebellum. Another role for NRG in the interactions between granule cells and Bergmann glia may be in the elongation of the Bergmann glial fibers since these cells become longer as the cerebellum becomes larger. Unfortunately, the role of NRG and erbB receptors in neuronal migration in vivo has not been tested by the inactivation of these molecules by homologous recombination in mice, since knockout animals die due to heart defects at E10.5, before neurogenesis and neuronal migration (Meyer and Birchmeier, 1995; Gassmann et al., 1995; Lee et al., 1995; Kramer et al., 1996). The role of these molecules could be tested either by tissue-specific NRG and erbB receptor knockouts, or by expressing the dominant-negative erbB4 receptor in a tissue-specific manner.

Our data suggest that neuronally produced NRG acts on the glial cells, inducing glial differentiation events that are necessary for the subsequent movement of neurons along glial processes. This could be mediated by the induction of transcription of glial genes whose products are critical for neuronal migration. This is supported by our finding that NRG induces the phosphorylation of the transcription factor CREB in cerebellar astroglia. Glial genes that could be induced by NRG include glial surface molecules, which are important for migration, such as Brain Lipid-Binding Protein, a glial protein that has been shown to be critical for granule cell migration (Feng et al., 1994) and whose expression is induced by neuron–glia contact (Feng and Heintz, 1995). However, it is also possible that NRG induces expression of other yet unidentified gene products that are important for migration.

Another mechanism by which NRG–erbB receptor signaling could regulate neuronal migration is the activation of a program of cell–cell reciprocal interactions between glia and neurons. For example, NRG may induce the expression of a glial gene that contributes to the generation of a retrograde signal, which then acts back on the neurons, inducing further steps of the migration program. NRG has been shown to be involved in such interactions in the peripheral nervous system. In developing sympathetic ganglia, NRG produced by neuroblasts promotes the expression of NT-3 by neighboring cells, which then promotes neuroblast survival and differentiation (Verdi et al., 1996). A similar regulatory loop has been proposed to take place at the developing neuromuscular junction. Several growth factors that are expressed by skeletal muscle cells (BDNF, NT-3, NT-4, and GDNF) induce NRG expression in spinal cord motoneurons (Loeb and Fischbach, 1997). Motoneurons, through NRG activation of muscle erbB receptors, would in turn induce the maturation of the neuromuscular junction. It seems likely that participation in reciprocal cell–cell signaling loops may be a central feature of NRG's function in the developing nervous system.

Our finding that NRG plays a critical role in mediating neuronal migration in the cerebellum may be relevant to neuronal migration in other regions of the developing nervous system. We have found that in the developing cortex, neuronal precursors express NRG while radial glial fibers contain erbB4 (Mason and Corfas, unpublished data). Therefore, NRG–erbB receptor signaling may play a role in neuronal migration in the developing

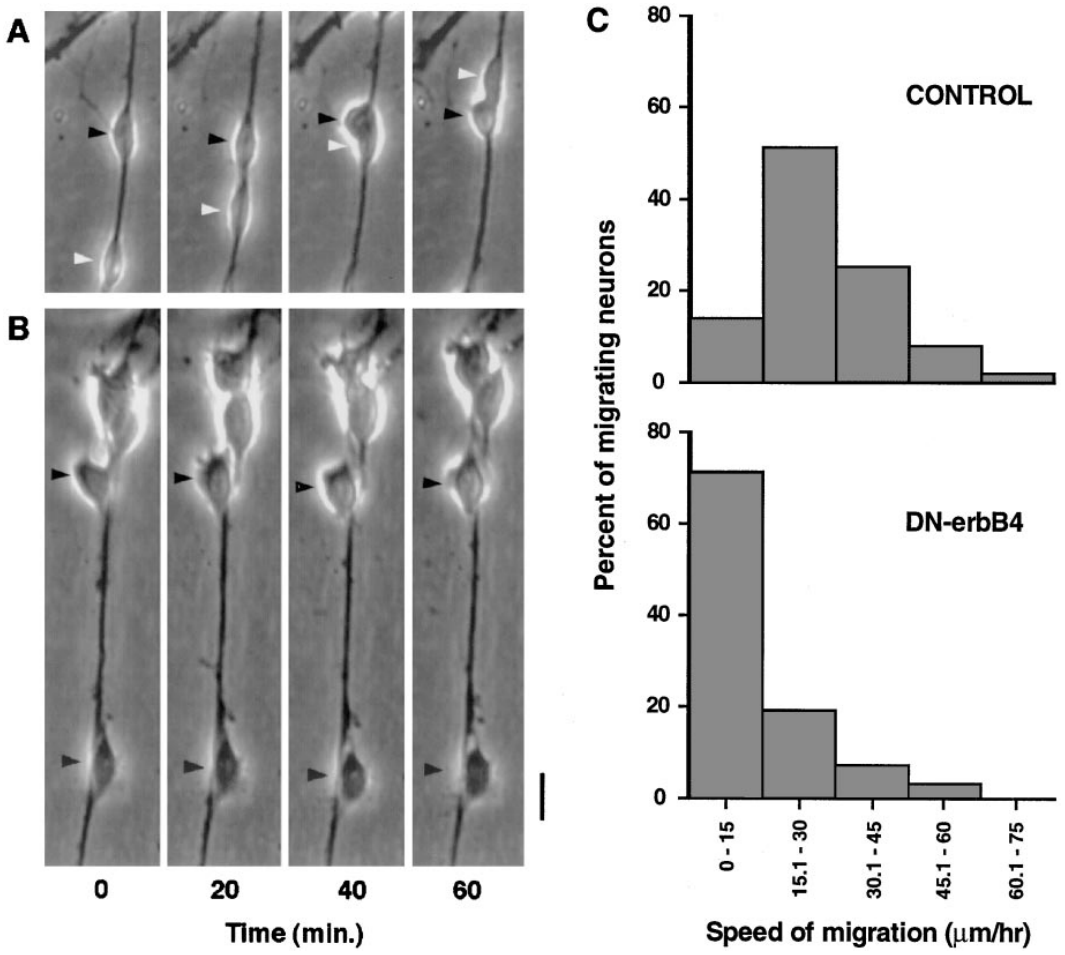

Figure 7. Glial Expression of DN-erbB4 Blocks Neuronal Migration

Migration of granule cells along control (A) and DN-erbB4 expressing (B) radial glial fibers. Time-lapse images are shown at 20 min intervals. The scale bar, 12 μm.

(A) Most granule cells move significant distances along the control fibers (white arrow), and only a few fail to move (black arrow).

(B) At least four granule cells are positioned on this glial fiber, and they exhibit only very slight movements during the period examined (examples labeled with black arrows).

(C) Percentage distribution of the speed of neuronal migration (μm/hr). Granule cells moving along control glial fibers migrate at an average rate of 27.5 ± 1.8 μm/hr (n = 49) while granule cells moving along DN-erbB4-expressing fibers migrate at an average rate of 15.9 ± 2.0 μm/hr (n = 31). The difference between the average rates of migration of the two groups is statistically significant ($P < 0.0001$).

cortex. However, the role of NRG in the interactions between cortical neuronal precursors and radial glia may be somewhat different than that in the cerebellum. While we found that NRG induces cerebellar astroglia to become radial, Hunter and Hatten (1995) showed that cortical astrocytes acquire radial glia identity in response to a yet unidentified factor, but not in response to NRG.

Other proteins expressed by granule cells are likely to work together with NRG to induce the neuronal migration program. Astrotactin, like some forms of NRG, is a transmembrane protein expressed by migrating granule cells. Disruption of astrotactin function leads to the inhibition of glial process extension and to decreased rates of neuronal migration along glial fibers (Edmondson et al., 1988; Fishell and Hatten, 1991), an effect that appears to be secondary to its effects on adhesion (Stitt and Hatten, 1990; Zheng et al., 1996). While NRG and astrotactin appear to have overlapping functions, the pattern of expression of these molecules is notably different: astrotactin is expressed only by postmitotic granule cells (Zheng et al., 1996), while NRG is expressed earlier in all cells in the EGL. This suggests that NRG–erbB receptor signaling acts earlier than astrotactin in the migration program. It is plausible that activation of erbB receptors in glia leads to the expression of an astrotactin receptor. Alternatively, glia, in response to NRG stimulation, could produce a factor that may then in turn stimulate the granule cells to produce astrotactin.

The mechanisms by which activation of erbB receptors lead to neuronal migration are still not defined. However, it is likely that specific intracellular proteins may underlie the effects of NRG–erbB receptor signaling on migration. Grb7, an SH2 domain protein, has been shown to bind tightly to erbB2 (Stein et al., 1994). Interestingly, the central domain of Grb7, a stretch of >300

amino acids, is highly homologous to the *F10E9.6/mig-10* C. elegans gene, which is involved in neuronal migration (Manser and Wood, 1990; Wadsworth and Hedgecock, 1996). Thus, it is possible that Grb7 or other members of the Grb family are involved in the NRG induction of neuronal migration in vertebrate cells. NRG and erbB receptors have been implicated in the motility of other cell types. NRG induces cell motility and chemotaxis of mature Schwann cells in vitro at doses submaximal for induction of cell proliferation (Mahanthappa et al., 1996). Recently, it has been shown that heparin-binding EGF-like growth factor stimulates chemotaxis of NIH 3T3 cells overexpressing erbB4 but not in cells overexpressing the EGF receptor (Elenius et al., 1997). NRG also induces chemo-invasion and chemotaxis of SKBr-3 breast cancer cells that overexpress erbB2 (Staebler et al., 1994). It has been suggested that erbB2 may regulate cell adhesion through its association with the cadherin–catenin complex (Ochiai et al., 1994). Thus, NRG's effects on neuronal migration may involve not only changes in gene expression but also postranslational modifications of cell-adhesion proteins or other glial proteins involved in the neuronal migration program.

It is important to note that there is a clear difference between the role of NRG in neuronal migration and in migration of other cell types. In all of the cases described above, NRG induces the migration or chemotaxis of the erbB receptor–expressing cells. In contrast, in neuronal migration, the erbB receptor–expressing cell (glia) does not move, but becomes supportive for the movement of the NRG-expressing cell (neuron). Therefore, the mechanism by which NRG induces neuronal migration may be different than the mechanisms involved in NRG induction of chemotaxis and migration of other cell types. Further characterization of the effects of NRG and erbB receptor signaling in astroglia should provide additional insight into the molecular mechanisms of neuronal migration.

Experimental Procedures

Cell Culture

Primary astroglial and neuronal cells were purified from P5–P7 rat cerebella (Sprague Dawley, Charles River) as described by Hatten (1985) with a few modifications. Briefly, the cerebella were excised, and the meninges were removed in ice-cold Dulbecco's phosphate-buffered saline (PBS, GIBCO BRL), under a dissecting microscope. The tissue was cut into small pieces and incubated in 1% trypsin (Sigma) and 0.1% deoxyribonuclease I (DNase, Worthington) in PBS for 10 mins at 37°C. After centrifugation, the pellet was triturated using successively decreasing bore size fire-polished glass Pasteur pipettes, in Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS, GIBCO BRL) and 0.1% DNase. The suspension was applied to a cold two-step gradient of Percoll (35%–60%, Pharmacia Biotech) in PBS. After a 3000 rpm centrifugation (Sorvall RT 6000B) at 4°C for 10 min, the astroglia are at the top of the gradient, and the neurons are in the 35%–60% interface. The cell fractions were recovered and washed once in PBS and twice in BME.

For glia cultures, the cells were resuspended in Basal Medium Eagle (BME, GIBCO BRL) with 10% horse serum (Sigma), 0.1% glucose (Sigma), 2 mM L-glutamine (GIBCO BRL), and 100 U/ml penicillin–100 μg/ml streptomycin (GIBCO BRL). Cells were preplated for 45 min on an uncoated 75 cm^2 flask to remove contaminating fibroblasts, and transferred to a 75 cm^2 flask coated with 25 μg/ml of Poly-D-Lysine (Collaborative) for 1 hr to allow astroglial attachment; the flask was rinsed and left in the same medium. Astroglial cells were used for no more than two passages.

For granule cell cultures, the cells were resuspended in BME with 10% horse serum, 5% fetal bovine serum (FBS, GIBCO BRL), 2 mM L-glutamine, 100 U/ml penicillin–100 μg/ml streptomycin, and 0.9% glucose. Cells were then preplated in 100 mm tissue culture dishes (Corning) coated with 25 μg/ml poly-D-Lysine three times for ~1 hr each. After each preplating period, the cells were lifted off by gently pipetting medium on the cells. The neurons were finally plated on poly-D-Lysine coated tissue culture dishes (50 μg/ml). In some circumstances, both astrocytes and granule cells were grown on 15 mm glass coverslips coated with 25 μg/ml of Poly-D-Lysine for the astroglia and 3 μg/ml Laminin and 15 μg/ml Poly-Ornithine for the granule cells.

L6 and COS-7 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin–100 μg/ml streptomycin. C2C12 cells were grown in the same medium as above but with 20% FBS. For C2C12 differentiation cells, the medium was changed to DMEM with 5% horse serum. All cells were incubated at 37°C with 5% CO_2 and 100% humidity.

Generation of the Dominant-Negative erbB4 Receptor

A truncated erbB4 receptor was generated by polymerase chain reaction with Vent DNA polymerase (New England BioLabs) using a full-length erbB4 receptor cDNA (Plowman et al., 1993) as the template. The upstream polymerase chain reaction primer (5′-TATAGCGGCCGCCACGGGATCTGAGACTTCC-3′) was designed to introduce a NotI site 5′ of the erbB4 sequence. The downstream primer (5′-TATACCCGGGTCACTTGTCGTCATCGTCTTTGTAGTCTGAGCCAAGGACTTTTAC-3′) was designed to introduce the flag epitope in frame following residue serine 726 of erbB4, followed by a stop codon and a SmaI site. After cutting it with NotI and SmaI, the polymerase chain reaction product was subcloned into the multiple cloning site of the pcDNA3 vector (Invitrogen) using XbaI (blunt-ended) and the NotI site. The identity of the clone was confirmed by sequencing.

Histology and Immunostaining

Rat pups (P0-P19) were sacrificed with CO_2 and fixed by cardiac perfusion with 4% paraformaldehyde in PBS. Cryosections (20 μm) were blocked with 3% BSA, 0.1% Triton X-100 in PBS for 1 hr at room temperature, followed by incubation with the primary antibodies diluted in the same solution at 4°C overnight. The sections were washed with PBS, and the detection procedure was carried out using the appropriate fluorescent secondary antibodies (from Jackson Immunoresearch Laboratories), or with alkaline phosphatase-conjugated anti-mouse secondary antibodies using NBT/BCIP as substrate (see Figure 4C; Boehringer Mannheim) for 1 hr at room temperature.

Cells in culture were immunostained following basically the same procedure after fixation with 4% paraformaldehyde in PBS at room temperature for 20 min. For the P-CREB visualization, a biotinylated horse anti-mouse IgG (H+L) (Vector) and the avidin–biotin–peroxidase complex (ABC Elite, Vector), with 3, 3′-diaminobenzidine as a peroxidase substrate were used.

The tissue sections and cells in culture were mounted with Vectashield mounting medium (Vector). Photographs were taken using the following microscopes: Nikon Microphot-FXA, Nikon Optiphot-2, Olympus 1X70, or a OZ confocal microscope (Noran Instruments).

The following primary antibodies were used: rabbit anti-NRG 1310 (Burgess et al., 1995), 1915 (Sandrock et al., 1995), HM94 (a rabbit antibody raised against a 27 amino acids peptide corresponding to aa 61–87 within the Ig domain of chick ARIA; Loeb, unpublished data), HM209 (a rabbit antibody raised against the entire extracellular domain of human NRG; Khurana, unpublished data), rabbit anti-erbB4 0615 (Zhu et al., 1995), rabbit anti-erbB2 (C-18), anti-erbB3 (C-17), rabbit anti-erbB4 (C-18) (Santa Cruz Biotechnology), mouse anti-human erbB4 (H4.77.16, NeoMarkers), rabbit anti-P-CREB (Ginty et al., 1993), mouse anti-Glial Fibrillary Acidic Protein (Boehringer Mannheim), rabbit anti-Glial Fibrillary Acidic Protein (Dako), and the MAb Q600, a granule cell–specific antibody (Gravel et al., 1987).

Recombinant NRG

Recombinant EGF-like domain of rat NDFβ_1 (NDF$\beta_{1[177–246]}$) (Wen et al., 1994) was used in all experiments.

Transient and Stable Transfections

Cells in culture were trypsinized and electroporated with 20 μg of plasmid DNA with an Electro Cell Manipulator 600 (BTX, 1050 μF, 280 V, 125Ω) in 4 mm gap cuvettes with 400 μl of HEPES-buffered saline pH 7.3. Cells were incubated at 37°C in 10 ml of tissue culture medium for 30 min, changed to fresh medium, and plated. To obtain stable L6 cell lines expressing DN-erbB4, clones were selected and maintained in media with G418 (500 μg/ml, GIBCO BRL). C2C12 cells were cotransfected with DN-erbB4 and a plasmid encoding a puromycin resistance gene (pBabe Puro; Morgenstern and Land, 1990). Clones were selected and maintained in medium with puromycin (2 μg/ml, Sigma). Positive clones were selected by immunocytochemistry with the anti-FLAG M2 antibody (Eastman Kodak Company) and with an anti-human erbB4 (H4.77.16, NeoMarkers).

Astroglial Morphology Assay

One day after transfection with DN-erbB4 or control plasmid (GFP), the astrocytes were treated either with NRG (1 nM), or with freshly dissociated cerebellar granules cells (1/5–10 neurons per astrocyte). Twenty-four hours later, the cells were fixed with 4% paraformaldehyde and labeled using the anti-human erbB4-specific monoclonal antibody (MAb) and a rabbit anti-Glial Fibrillary Acidic Protein. Nuclei were stained with Hoechst 33342 (Molecular Probes), washed, and mounted. For quantitation of glial morphology, a blind experimental design was used. Random fields were selected by a different person than the investigator. First, the morphology of the cells in each field was assessed by looking at the GFAP staining, and only then the cells expressing the transgene were identified. In neuron–glia cocultures, the number of neurons in contact with every glia was also obtained. Astrocytes were considered to be radial when they had a thin process longer than 50 μm. This criterion was based on previous studies (Hatten, 1985) and our own preliminary observations.

Immunoblotting

Tissue culture cells were lysed with 2X DTT sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 6% sodium dodecyl sulfate, 0.1 mg/ml bromophenol blue, and 100 mM Dithiothreitol). For the erbB4 blots, plasma membranes were prepared according to Mason et al. (1990), and the protein concentration was estimated by the method of Bradford (1976). Aliquots were resolved in SDS–polyacrylamide gels, and transferred to Immobilon-P (polyvinylidene difluoride) membranes (Millipore). The transferred proteins were probed with one of the following antibodies: mouse monoclonal anti-phosphotyrosine antibody (4G10), mouse anti-FLAG M2 MAb (Eastman Kodak Company), rabbit anti-erbB4 (C-18, Santa Cruz Biotechnology), and a rabbit anti-NRG (1915, Amgen). Blots were incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Boehringer Mannheim), developed with Renaissance (DuPont NEN), and exposed to autoradiography film (Reflection, DuPont NEN).

Time-Lapse Video Microscopy and Quantification of Migration

Astroglia were grown in low density cultures in 35 mm tissue culture dishes for 1 day after transfection. Freshly dissociated granule cells were added to the glia at a ratio of 5–10 neurons per glial cell, the cocultures were incubated overnight, and then time-lapse imaging was performed using Image-1 imaging software (Universal Imaging) and a Nuvicon camera (MTI, Series 68). HEPES (10 mM) was added to the media, and media was covered with a layer of mineral oil to prevent evaporation. Dishes were placed in a Leiden microincubator (Ince et al., 1983; Medical Systems Corp.) and kept at 37°C throughout the experiment (Narishige MS-C temperature controller). Cells were observed with an inverted microscope (Leitz Fluovert) under phase contrast illumination. Cells were chosen for imaging based on only three criteria: (1) the glial cell was transfected (indicated by GFP), (2) the glia had a radial process >50 μm, and (3) granule cells, identified by their characteristic cell body and nucleus size and shape, were positioned on this radial process. Once cells were chosen, they were immediately imaged for 50–90 min, with images being acquired every 10 min. The position of granule cells along glial fibers was tracked using Image-1 imaging software, and distances and speed of migration were determined. Speed of migration was calculated as the average rate of migration during the duration of the experiment.

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