Endogenous Neuregulin Restores Radial Glia in a (Ferret) Model of Cortical Dysplasia

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Radial glia are integral components of the developing neocortex. During corticogenesis, they form an important scaffold for neurons migrating into the cortical plate. Recent attention has focused on neuregulin (NRG1), acting through erbB receptors, in maintaining their morphology. We developed a model of developmental radial glial disruption by delivering an antimitotic [methylazoxy methanol (MAM)] to pregnant ferrets on embryonic day 24 (E24). We previously found that normal ferret cortex contains a soluble factor capable of realigning the disorganized radial glia back toward their normal morphology. Characterization of the reorganizing activity in normal cortex demonstrated that the probable factor mediating these responses was a 30–50 kDa protein. To test whether this endogenous soluble factor was NRG1, we used organotypic cultures of E24 MAM-treated ferret neocortex supplemented with the endogenous factor obtained from normal cortical implants, exogenous NRG1β, antibodies that either blocked or stimulated erbB receptors, or a soluble erbB subtype that binds to available NRG1. We report that exogenous NRG1 or antibodies that stimulate erbB receptors dramatically improve the morphology of disrupted radial glia, whereas blockade of NRG1-erbB signaling prevents the radial glial repair. Our results suggest that NRG1 is an endogenous factor in ferret neocortex capable of repairing damaged radial glia and that it acts via one or more erbB receptors.

Key words: neocortical development; cerebral cortex; erbB receptors; MAM; organotypic culture; schizophrenia

Introduction

Nearly two decades ago, Hatten (1985) showed that contact with granule neurons induces cerebellar astroglia to adopt the morphology of radial glia, suggesting that neuronal derived signals are important for radial glia development. In a later study, Hunter and Hatten (1995) raised the possibility that this process may be regulated by a soluble “radialization factor.” Through a series of cell culture, morphological, and biochemical studies, they showed that embryonic neocortical cells produce a soluble substance of 50–60 kDa molecular weight (MW) (called RF60), which regulates radial glial and astrocytic morphology. Previous studies in our laboratory also strongly suggested that one or more radializing factors may be present during the period that radial glia are needed as a scaffold for cells migrating into the neocortex. We reported that injection of the antimitotic drug methylaazoxy methanol (MAM) into pregnant ferrets early during corticogenesis [embryonic day 24 (E24)] resulted in a cluster of symptoms that included disrupted migration, altered distribution of Cajal Retzius cells, disorganized radial glial cells, and the premature differentiation of radial glia into astrocytes (Hasling et al., 2003).

Furthermore, we observed that the morphology of the radial glia could be “rescued” in tissue culture either by coculture with normal cortical slices or by administration of medium conditioned by normal cortical tissues. We subsequently characterized this cortical-derived “factor” as a diffusible protein of nominal molecular weight of ~66 kDa (Gierdalski and Juliano, 2003).

Looking for potential candidates to be responsible for the cortically derived activity, we identified NRG1, a soluble factor that signals through the erbB receptor tyrosine kinases erbB2, erbB3, and erbB4 (Marmor et al., 2004), which influences the morphology of radial glia in the cerebellum and the cortex. Rio et al. (1997) showed that NRG1 is expressed by migrating cerebellar granule neurons (whereas Bergmann glia express erbB receptors) and demonstrated that NRG1-erbB signaling mediates the conversion of cerebellar astroglia into radial glia in response to neuronal contact. Anton et al. (1997) found that NRG1 is also expressed by migrating neocortical neurons and that in vitro NRG1 promotes the maintenance of radial glia morphology in cortical imprint cultures (Anton et al., 1997).

In the present study, we demonstrate that NRG1 is a substance endogenous to normal ferret cerebral cortex and is capable of repairing disrupted radial glial morphology. We show that medium conditioned by organotypic cultures of normal ferret cortex contains NRG1 and that NRG1, or antibodies activating erbB receptors, repairs the morphology of radial glia after MAM treatment. Furthermore, we found that reagents blocking NRG1-erbB signaling also block the ability of medium conditioned by normal...
cortical slices to repair the morphology of MAM-treated radial glia. Together, these results show that NRG1 is responsible for at least part of the activity that repairs radial glia disrupted by MAM.

Materials and Methods

MAM treatment and organotypic cultures. Timed pregnant ferrets obtained from Marshall Farms (New Rose, NY) were anesthetized with isoflurane (3%) and nitrous oxide (0.05%). They were injected intraperitoneally with 16 mg/kg MAM on E24 as described by Noctor et al. (1999). Brain slices from postnatal day 0 (P0) ferret kits were prepared as described previously (Giedralski and Juliano, 2003; Hasling et al., 2003). In brief, kits were anesthetized with pentobarbital Na (50 mg/kg, i.p.), and the brains were removed and sliced at 500 μm in thickness using a tissue chopper. The most rostral and caudal portions of the brain were discarded, and the cultures were prepared predominantly of the presumptive parietal cortical. Each slice was placed in a 70 μm nylon mesh cell strainer (Becton Dickinson-Falcon, Bedford, MA), which was then positioned in a six-well tissue culture plate (Becton Dickinson-Falcon). In some cases, the cortical plate was dissected from corresponding slices of normal cortex at P0, and the explants were placed adjacent to the E24 MAM-treated slice as reported by Hasling et al. (2003). Neurobasal medium (supplemented with B27 and N2; Invitrogen, Carlsbad, CA) with the addition of gentamicin and L-glutamine was placed in each well to allow the slice to cover the slice as described by Stoppini et al. (1991). The slice cultures were maintained in an incubator (37°C; 95% O2/5% CO2) for the duration of the culture period.

To assess the ability of recombinant or endogenous NRG1 to restore the morphology of radial glia and axons through ErbB receptors, we used organotypic cultures of neonatal ferret cortex. Slices of cortex were obtained from normal or E24 MAM-treated kits at P0. They were placed in culture alone, as controls, treated with neuregulin or other substances, or in coculture of MAM-treated slices with isochronic explants from normal cortex. The cultures received additions of human recombinant NRG1β (1 nM; R & D Systems, Minneapolis, MN), anti-ErbB3 blocking antibody, anti-ErbB4 activating antibody (20 μg/ml), and subsequently analyzed.

Analysis of radial glial morphology. The label resulting from the dextran injections was quantified by measuring the angular deviation of labeled processes. Each dextran injection was digitally imaged at 25× to allow individual processes to be traced and converted to line drawings. To trace entire glial processes labeled by the dextran injections, several focal planes for each injection were imaged and collapsed. The angles were measured along a chord of each radial glial process, as described previously in detail (Hasling et al., 2003). The average of the absolute differences represented the mean angular deviation of each injection. The mean angular deviation was compared for statistically significant differences using an ANOVA followed by Tukey’s test for multiple comparisons between conditions.

Western blots. The fractionation of the conditioned medium was conducted as described by Giedralski and Juliano (2003). In brief, after 2–3 d of culturing cortical slices from normal P0 ferrets in serum-free medium (Neurobasal plus N2 plus B27 plus gentamicin/L-glutamine; Invitrogen, Carlsbad, CA), the medium was collected and centrifuged while passing through a centrifugal filter device (Amicon Centriprep YM; Millipore, Billerica, MA) with cutoff limits of 50, 30, and 10 kDa MW, consecutively. The fractions were then concentrated and buffer-exchanged three times by centrifugation, washing with replacement TBS buffer (20 mM Tris HCl, 20 mM NaCl, pH 7.2; containing Complete Protease Inhibitor Cocktail tablets; Roche, Indianapolis, IN) using filter devices (Amicon Centricron YM-3; Millipore, Billerica, MA). All centrifugations were done at 4°C. Filtrates were collected and immediately frozen at −80°C.

The samples of concentrated fractions (in equal amounts equivalent to one original cultured slice; i.e., one slice equivalent) were mixed with 4× NuPAGE LDS Sample Buffer (Invitrogen) and 10× NuPAGE Sample Reducing Agent (Invitrogen), boiled for 10 min, loaded onto NuPAGE BisTris 10% minicasette, run in 3–(N-morpholino)propanesulfonic acid SDS running buffer with the addition of NuPAGE antioxidant. The gel was transferred onto a nitrocellulose membrane (0.45 μm pore; Millipore, Bedford, MA) using XCell SureLock. The membrane was incubated overnight at 4°C in blocking buffer (PBS, 0.5% casein, 0.1% Tween 20) and then incubated with an anti-neuregulin monoclonal primary antibody Ab-1 (MS-272; 1:100 in blocking buffer; Neomarkers, Fremont, CA), or the neuregulin inhibitor IgB4, a chimeric protein composed of the extracellular domain of erbB4 and the Fc portion of human IgG1 (Dong et al., 1995).

Injections of fluorescent dextrans. After being in culture for 2 d, each organotypic culture was removed from the incubator and placed in a chamber used to maintain living slices as described previously (Juliano et al., 1996). Each culture received an iodophoretic injection (4 μl; alternating positive current for 3 min) of fluorescently labeled dextrans (Molecular Probes, Eugene, OR) (we primarily used Fluororuby) into the intermediate zone at a depth of 50–100 μm, as described by Noctor et al. (1999). The injections were made using a glass pipette with a tip diameter of ~20 μm. After an additional 5–8 h of incubation in the chamber at room temperature, the slices were placed in 4% buffered paraformaldehyde for ~12 h. Before mounting on subbed slides, the slices were counterstained with bisbenzimide trihydrochloride (Sigma, St. Louis, MO) and subsequently analyzed.

AerB phosphorylation assay. The activity of the fractions was assessed on L6 rat myoblast cells by analysis of the induction of p185 tyrrosine phosphorylation, as described by Corfas et al. (1993). Briefly, cells were grown in DMEM containing 10% fetal bovine serum, Pen/Strep, and L-glutamine. Cells were plated in 24-well plates and treated with NRG1 (1 μg/ml) or conditioned media fractions for 5 min. Cells were washed with cold PBS on ice, lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 μg EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM Pefabloc), and centrifuged for 10 min at 1000 × g. Lysates were incubated overnight with erbB2 and erbB3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and immunoprecipitated with protein A/G beads (Pierce) for 1 h. Samples were then washed three times with lysis buffer before the beads were resuspended in SDS sample buffer before boiling for 2 min. Samples were loaded onto 5% polyacrylamide–SDS gels. For electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblotting using the monoclonal phosphophorysine antibody 4G10 (a gift from O. Gjoerup and T. M. Roberts, Dana-Farber Cancer Institute, Boston, MA). The blots were then incubated with HRP-conjugated anti-mouse secondary antibody and developed using enhanced chemiluminescence kit (Pierce, Rockford, IL) according to the manufacturer. The blots were then exposed to Hyperfilm ECL (Amersham Biosciences, Little Chalfont, UK). Developed films were digitized using LS-1000 Imaging System (Fujifilm, Tokyo, Japan).

Results

Medium conditioned by ferret cortical slices contain NRG1

To assess whether NRG1 was present in the neonatal ferret cortex, medium conditioned for 48 h by organotypic cultures of normal cortical slices was fractionated into aliquots of different molecular weight by centrifugation. Then, the fractions containing molecules of 10–30 and 30–50 kDa nominal molecular weight were subjected to Western blot analysis with antibodies against the extracellular domain of NRG1. We previously demonstrated that fractionated conditioned media of nominal molecular weight 30–50 kDa could restore radial glia disrupted by MAM treatment to normal morphology (Giedralski and Juliano, 2003). The blots shown in Figure 1A show the existence of soluble NRG1 in the conditioned media obtained from normal (control) slices but not in the media obtained from E24 MAM-treated
Activation of erbB receptor signaling is sufficient for repair of radial glia morphology

To further test the roles of NRG1-erbB signaling in radial glia repair and to test the effects of erbB4, the other NRG1-binding receptor in this process, we asked whether activation of erbB4 receptor signaling independent of NRG1 could also repair radial glia morphology. E24 MAM-treated slices were treated with an erbB4 antibody that binds and activates the receptor. This treatment was sufficient to cause glial processes to elongate, reducing the angular deviation of radial glial fibers toward normal levels; the angular deviation values after erbB4 activation are not signifi-
cultures. The reactivity is of slightly higher molecular weight than we expected, ~66 kDa. The range of molecular weight is shown in Figure 1C, which illustrates a silver stain obtained from a gel of this media fraction.

To determine whether the fractions of cortical conditioned medium that can repair the morphology of radial glia after MAM treatment contain active NRG1, we tested whether they induce the activation of erbB receptors. L6 muscle cells, which express erbB2 and erbB3 receptors, were stimulated with the inactive (10–30 kDa) or active (30–50 kDa) conditioned media fractions from normal cortex and then subjected to phosphotyrosine Western blot analysis. The muscle cells were also stimulated using the normal active fraction with a soluble form of erbB4 added and with the 10–30 and 30–50 kDa fractions obtained from MAM-treated animals. The soluble erbB4 would be expected to bind to NRG1 and block its function. Only the active fraction obtained from normal conditioned medium induced tyrosine phosphorylation of a 185 kDa band, corresponding to the molecular weight of the erbB receptors (Fig. 1D). Together, these results show that NRG1 is present in the 30–50 kDa conditioned media fraction obtained from normal ferret cortex but not in MAM-treated cortex. In addition, the ability of the 30–50 kDa fraction to phosphorylate L6 muscle cells was blocked by the addition of soluble erbB4.

Recombinant soluble neuregulin repairs radial glia

To assess whether NRG1 could be responsible for elongating the disrupted radial glia in E24 MAM-treated cortex, organotypic cultures from P0 E24 MAM-treated cortex were maintained in the presence or absence of 1 nM NRG1 for 2 d. Slices were then injected with FluoroRuby, which selectively labels a small population of radial glial cells and their processes (Noctor et al., 1999), and the angular deviation of radial glia was measured. Addition of NRG1 to the cultures had a strong radializing effect, as indicated by the straightening and elongation of radial glial morphology in E24 MAM–treated cortex compared with the morphology of cultures without added NRG1 (Fig. 2B–E). An example of the elongation effect of using the fraction of normal conditioned media of 30–50 kDa in an E24 MAM–treated slice is shown in Figure 2A. To show this effect, the media fraction was adhered to fluorescent microspheres, which were injected into the organotypic culture.

The radializing effect of NRG1 is quantified in Figure 3, which shows that normal radial glia have a low angular deviation, with well aligned fibers (black bar). The angular deviation of E24 MAM–treated slices is high, with the glial fibers arrayed at many angles (Fig. 3, white bar), but when NRG1 is added to MAM–treated tissue, the angular deviation is no longer significantly different from the normal angles (gray bar).

To further assess the effects of NRG1 on the repair of MAM–treated radial glia, we tested whether blockade of NRG1 binding to erbB3 would affect the ability of this factor to repair radial glia morphology. E24 MAM–treated organotypic cultures were treated with NRG1 for 48 h in the presence or absence of the blocking erbB3 antibody. Although radial glial morphology in slices treated with NRG1 had improved morphology, addition of the erbB3 blocking antibody abolished the NRG1 effect (Figs. 3, 4). These results show that NRG1 is sufficient to induce the radial glia repair of MAM–treated slices and that erbB3 may be involved in this process.
additives to the normal medium. Our previous studies demonstrated that after 2 d in culture, the E24 MAM-treated cortex cultured with normal cortical explants showed the typical elongation and improvement that occurs in the presence of a normal cortical plate (Hasling et al., 2003). The presence of the IgB4 receptor, however, completely abolished these effects (Figs. 3, 4).

**Discussion**

Is neuregulin present in conditioned medium of a normal ferret?

Our data suggest that neuregulin is endogenous to neonatal ferret cortex and acts via erbB receptors to maintain an elongated morphology in radial glial cells. The substance we previously identified in conditioned medium obtained from neonatal ferret cerebral cortex acts similarly to exogenous NRG1. In the current study, the addition of NRG1 to cultures with disrupted radial glia results in dramatic improvement of their morphology, as does the addition of the identified fraction (Gierdalski and Juliano, 2003). The activity of the conditioned media fraction is concentrated in a nominal MW 30–50 kDa, within the range of NRG1 molecular weight (Gierdalski and Juliano, 2003). Western blots reveal that NRG1 is present only in the media fraction that contains the radial glia-reorganizing activity. In addition, the active fraction is capable of acting in a manner similar to NRG1 by causing erbB receptor tyrosine phosphorylation in muscle cells. We further observed that blockade of endogenous NRG1 by a soluble erbB4 receptor also prevents its action in our model of disrupted radial glia. These observations strongly indicate that NRG1 is present in the developing ferret cortex, is likely to be the factor we previously identified, and acts to elongate radial glia.

NRG1 is a multiform protein and an active integral membrane proprotein. Alternative splicing of a single gene yields three different types (I, II, and III) that differ in the existence of specific domains, glycosylation sites, and molecular topology (cf. Falls, 2003). Furthermore, it has been shown that type I and, possibly, type II of the NRG1 molecule can be cleaved proteolytically by metallocproteinases 17 and 19 containing a disintegrin and metalloprotease domain (ADAM17 and ADAM19) (Shirakabe et al., 2001; Montero et al., 2002) and an extracellular portion released to act as a soluble agent (cf. Buonanno and Fischbach, 2001; cf.
Neuregulin acts as a radialization factor through ErbB3 and ErbB4 receptors

Members of the erbB receptor family are important in the regulation of cell growth and differentiation (cf. Neve et al., 2001). These receptors interact with each other forming dimers in a seemingly nonrandom manner (cf. Rubin and Yarden, 2001). The content of a dimer has great impact on its signal transduction abilities, with heterodimers being more potent (Wang et al., 1998). In our model, direct application of NRG1 results in radial glial remodeling, whereas blockade of endogenous NRG1 (by soluble erbB4) results in failure of radial glial elongation. The “rescue” of radial glia morphology seems to be mediated via both erbB3 and erbB4, because activation of erbB4 causes an extension of radial glia and blockade of erbB3 prevents radial glial transformation. The potency of these receptors may be increased by dimerization, as described above. Because the kinase domain of the erbB3 receptor is catalytically inactive, this implies that the result seen here is caused by heterodimerization with erbB4 (Citri et al., 2003; Marmor et al., 2004). Our results also suggest that erbB3 and erbB4 together are sufficient for radializing activity. We cannot rule out that erbB2 is also important in this process, because these receptors are also present in the cerebral cortex and often dimerize with other erbB receptors, but they were not tested in our assay. Interestingly, Schmid et al. (2003) demonstrated, using a dominant-negative erbB2 construct, that NRG1-erbB2 signaling can influence the morphology and maintenance of radial glia in the mouse.

Conclusion

Once neuronal migration is complete, some radial glia transform into astrocytes (Voigt, 1989; Culican et al., 1990; deAzevedo et al., 2003). This transformation has been reported to be bidirectional, in that astrocytes can revert to an elongated radial glia-like state both in vitro (Hatten, 1985; Hunter and Hatten, 1995; Soriano et al., 1997; Leprince and Chanas-Sacre, 2001) and in vivo (Leavitt et al., 1999). NRG1 appears to be important to this reversion, because it can induce cerebellar astrocytes in culture to adopt a radial glia phenotype (Rio et al., 1997). In this study, we demonstrate that NRG1 induces partially differentiated radial glia to revert to an elongated state in organotypic cultures. Thus, NRG1 may be involved in the other instances in which astrocytes transform into radial glia in vivo.

For several years, radial glia have been accepted as neural progenitor cells (Malatesta et al., 2000; Noctor et al., 2002; Tamamaki et al., 2001). NRG1 may play a role in the function of radial glia as such progenitors, because with maturation and
conversion into astrocytes, they appear less able to generate neurons. NRG1-erbB signaling is clearly part of a mechanism that maintains the radial glial phenotype as long as needed for proper development, including their function as progenitor cells.

NRG1-erbB signaling has received particular recent attention, because the genes encoding neuregulin and receptors have been implicated in predisposition to schizophrenia (Moises et al., 2002; Stefansson et al., 2002; Corfas et al., 2004). The results from the present study provide one mechanism that could contribute to a deficit seen in schizophrenia: failure of migration in various cortical regions (Akbarian et al., 1996; Rioux et al., 2003). Reduced NRG1 contributes to disruption of radial glia, which in turn influences the ability of neurons to migrate properly into the cerebral cortex. Additional analysis may clarify the brain regions involved and whether NRG1 has a precise role in this process.

References


