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Neonatal Oligodendrocytes Contain and Secrete Neuregulins In Vitro

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Abstract: The factors that influence the development of oligodendrocyte (OLG) progenitors into mature OLGs remain elusive. Recent evidence has suggested that neu differentiation factor (NDF), which is a member of the neuregulin family of growth factors, influences the development of glial cells, including Schwann cells, astrocytes, and OLGs. Neurons are postulated to be the source of neuregulins, because neurons closely interact with these glial cells during development. In this report, we have identified the mRNA for both isoform families of NDF in cultured neonatal (immature) OLGs. We have also demonstrated that cultured neonatal OLGs contain and secrete NDF protein. These data raise the possibility that NDF could be used in an autocrine/paracrine loop by neonatal OLGs during development for survival, proliferation, and/or differentiation. **Key Words:** Neuregulin—Autocrine—Oligodendrocyte—Neu differentiation factor.
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Isolation of ligands that elicit tyrosine phosphorylation of $p185^{erbB2}$ (also known as erbB-2/neu) led to the identification of neu differentiation factor (NDF), glial growth factor, acetylcholine receptor-inducing activity (ARIA), and heregulin (Holmes et al., 1992; Peles et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993). Molecular cloning of these different ligands indicated that they constituted a related family of growth factors, which were named neuregulins (NRGs) (Marchionni et al., 1993; Peles and Yarden, 1993). Two major isoform families of NRGs (termed NRGα and NRGβ) have been identified based on sequence differences of approximately six to eight amino acids occurring between the fifth and sixth cysteine residues of the epidermal growth factor-like domain (Wen et al., 1994). The majority of NRG molecules are synthesized as membrane-bound precursors termed proNRGs, which are processed subsequently to produce the soluble, secreted forms of NRGs (Wen et al., 1992; Burgess et al., 1995). Both proNRGs and secreted NRGs are biologically active, resulting in possible paracrine/autocrine and juxtacrine (cell-to-cell) signaling between cells expressing the appropriate receptors (Massagué and Pandiella, 1993).

Individual members of the NRG family demonstrate a wide array of biological functions in the developing CNS. It has been demonstrated that ARIA increases the number of oligodendrocyte (OLG) progenitor cells extending sheet-like processes that are characteristic of more mature OLGs (Vartanian et al., 1994). Conversely, glial growth factor has been shown to enhance the proliferation and survival of OLG progenitors while inhibiting the differentiation of these same cells (Canoll et al., 1996).

Conflicting reports have also been published concerning the expression of NRG mRNA in OLGs. For example, Pinkas-Kramarski et al. (1994) using Southern blot analysis following reverse transcription (RT)–PCR amplification of cDNA for NRGs in neonatal OLGs reported no evidence of NRGs in OLGs. In contrast, Vartanian et al. (1994) using in situ hybridization on sections from both embryonic and postnatal rat brain reported that the subventricular zone (a region known to contain developing OLGs) was positive for NRG mRNA.

In this report, we demonstrate that cultured neonatal OLGs obtained from postnatal day 2 rat pup brains contain mRNA for both NDFα and NDFβ. We also demonstrate that neonatal (immature) OLGs express and secrete NDF protein. These results are consistent with the view that endogenously produced NRGs may play a role in the development of OLGs. For example, NRGs produced by OLGs during development could promote survival of OLGs, as well as stimulating the

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Abbreviations used: ARIA, acetylcholine receptor-inducing activity; NDF, neu differentiation factor; NRG, neuregulin; OLG, oligodendrocyte; RT, reverse transcription.

proliferation and/or differentiation of OLG progenitors or neonatal OLGs.

MATERIALS AND METHODS

Establishment of primary cultures of neonatal OLGs

Neonatal OLGs were obtained from the cortices of postnatal day 2 rat pups according to the methods of McCarthy and de Vellis (1980). In brief, cerebral cortices were isolated, cleaned of meningeal tissue, and minced. Following digestion with acetylated trypsin (Sigma) and filtration through Nitex 130 and Nitex 35 filters, cell filtrate was maintained in OLG medium (described by Chen and DeVries, 1989) with 10% fetal calf serum for 2 days, at which time cultures were shifted to OLG medium containing 10% Nu-Serum I (Collaborative Research). After 7 days, cultures were shaken overnight at 350 rpm and subjected to differential adhesion on plastic Petri dishes to remove contaminating astrocytes. The differential adhesion step results in astrocyte contamination of <5% (data not shown). OLGs were collected and replated on poly-D-lysine-coated coverslips or six-well clusters in OLG defined medium (described by Chen and DeVries, 1989).

Immunocytochemistry of cultured neonatal OLGs

OLGs were plated at a density of 25×10^3 cells/12-mm coverslip (Fisher Scientific) in OLG defined medium containing 0.1% horse serum. The next day, cells were rinsed and fresh OLG defined medium was added. Cultures were maintained for 5 days and processed for immunocytochemistry as previously described (Tzeng et al., 1995). After incubation overnight at 4°C with a pan-specific monoclonal anti-NDF antibody 5D6A (5 μg/ml; Amgen, Inc.), which recognizes both NDF isoform families, the cells were incubated with goat anti-mouse fluorescein-conjugated secondary antibodies (Boehringer Mannheim) to visualize cells. Cells were photographed with a Leica Leitz DMRB microscope equipped with a Leica Wild MPS 48/52 camera system.

Western blotting of neonatal OLGs and conditioned medium from cultured neonatal OLGs

OLGs were plated in six-well clusters (35 mm diameter; Costar) at a density of 25×10^4 cells/well in OLG defined medium. After 5 days, proteins were extracted, subjected to electrophoresis, and transferred to polyvinylidene difluoride membrane (PolyScreen, Du Pont) as previously described (Raabe et al., 1996). Membranes were blocked with 5% milk in phosphate-buffered saline containing 0.1% Tween-20 for 30 min and incubated overnight at 4°C with a polyclonal anti-NDF antibody 1915 (3 μg/ml; Amgen), which recognizes both isoform families of NDF. The separated proteins were visualized using enhanced chemiluminescence protocols (Du Pont) as previously described (Raabe et al., 1996).

Conditioned medium was collected from cultured neonatal OLGs plated in six-well clusters (Costar) at a density of 30×10^3 cells/well. After 5 days, serum-free OLG conditioned medium (~10 ml) was collected from the wells and concentrated using centrifugal filters (Ultrafree-15, Millipore). Conditioned medium was fractionated using heparin-affinity chromatography and analyzed for NDF using anti-NDF antibody 1915 as previously described (Raabe et al., 1996).

RT-PCR of neonatal OLGs

OLGs were plated at a density of 25×10^4 cells/well of a six-well cluster in OLG defined medium. After 5 days, total RNA was isolated, reverse-transcribed, and subjected to a nested PCR strategy using primers designed to identify either NDFα or NDFβ. Two microliters of each cDNA was amplified with the following primers for 32 cycles at an annealing temperature of 58°C. First, primers designed to amplify all NDF isoforms were derived from the $NDF\alpha_{2B}$ sequence (accession no. U02316). These primers corresponded to a sense primer starting at bp 812 (5′-CTCAGTTTCAACAGAAGGCGCAAAC-3′) and an antisense primer starting at bp 1,221 (5′-GTTTGGGTGGTGAGGCCCATTCGC-3′), yielding a 432-bp product. One microliter of a 1:100 dilution of the 432-bp PCR product was then used in a second PCR reaction using a common internal sense primer starting at bp 888 (5′-TGTGCGGAGAAGGAGAAAACTTTC-3′) specific for all NDF isoforms in combination with either an antisense primer starting at bp 1,017 (5′-TTGGGTTTGGACTTTCATGGGTAC-3′) specific for the NDFα isoform family or an antisense primer starting at bp 1,008 (5′-GAAGCTGGCATTAGGTAGTTTTG-3′) specific for the NDFβ isoform family (accession no. U02318). The use of these two isoform-specific primers should yield 153-bp and 144-bp products, respectively.

RESULTS

We have demonstrated that neonatal OLGs contain NDF protein by using monoclonal antibody 5D6A designed to detect either NDFα or NDFβ. Neonatal (immature) OLGs contained NDF immunoreactivity after 5 days in culture (Fig. 1A, arrows). The NDF immunoreactivity was present in the cell body (arrows) as well as the processes (arrowheads) of the neonatal OLGs (Fig. 1A). Incubation of the OLGs with either no primary antibody (Fig. 1C) or primary antibody first incubated with recombinant NDF protein (data not shown) resulted in negligible background staining of cultured neonatal OLGs.

We confirmed the presence of NDF in neonatal OLGs by using western blot analysis of OLG lysates with the anti-NDF antibody 1915, which recognizes both isoform families of NDF. The use of monoclonal antibody 5D6A for immunocytochemical analyses and polyclonal antibody 1915 for western blotting analyses was due to the more intense staining pattern of 5D6A on cultured cells. OLG lysates probed with antibody 1915 contained immunoreactive polypeptides at 80, 45, 35, and 25 kDa (Fig. 2A, arrows). It should be noted that 5D6A recognizes similar polypeptides on western blots as does 1915. These different molecular weight polypeptides are likely to correspond to different isoforms of NDF. For example, the 80-kDa polypeptide could correspond to a proNDF molecule residing in the cell membrane of OLGs. The 45-kDa polypeptide possibly represents the mature, secreted form of NDF as previously described in numerous other

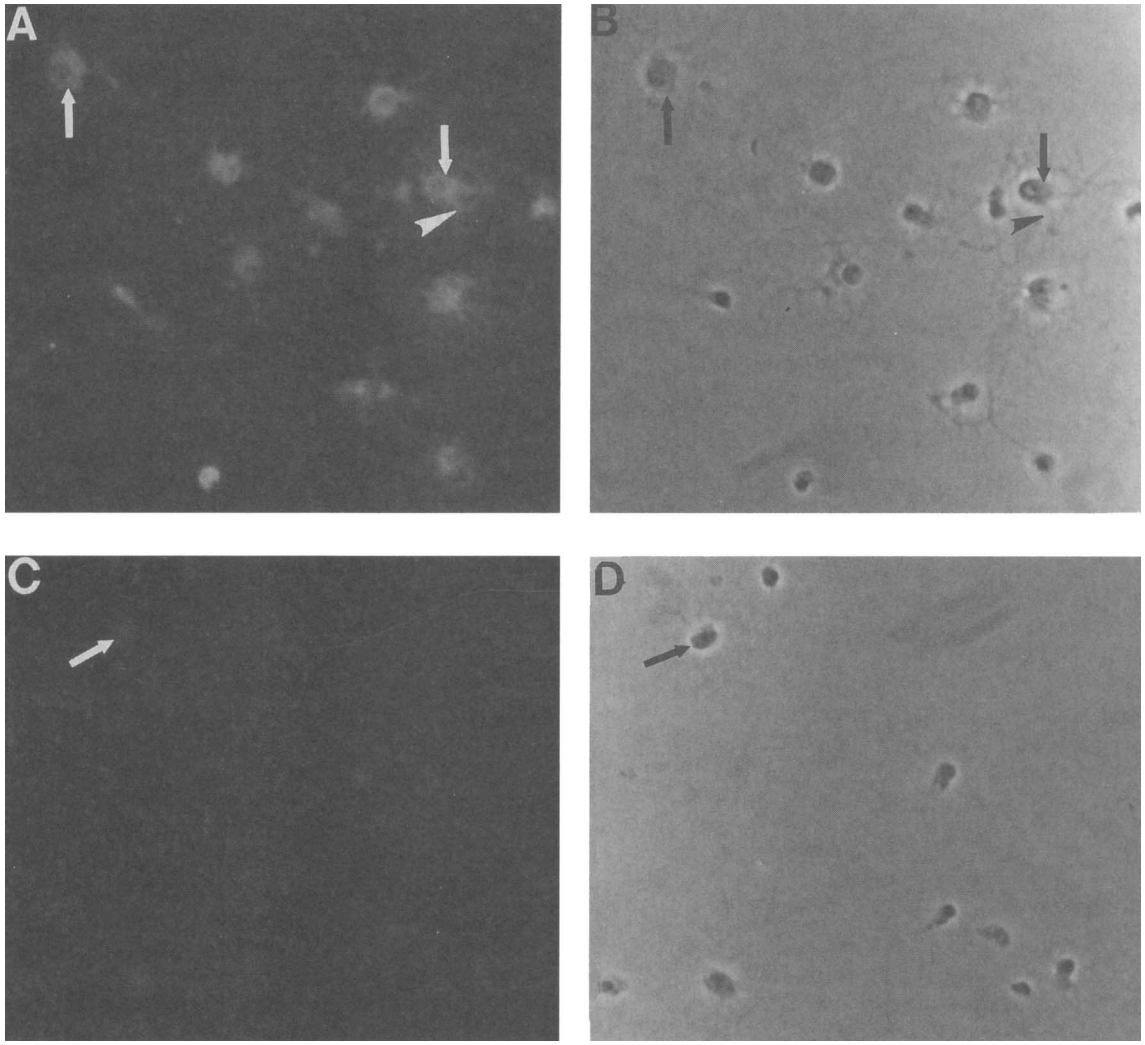

FIG. 1. Fluorescence (**A** and **C**) and phase (**B** and **D**) photomicrographs of neonatal OLG cultures. In neonatal OLGs immunostained with anti-NDF antibody 5D6A (A and B), immunoreactivity was observed throughout the cell body (arrows) and the processes (arrowheads) of the OLGs. In neonatal cultures not treated with primary antibody 5D6A (C and D), background immunoreactivity is negligible. These results are representative of experiments repeated at least three times. ×920.

studies. The 35-kDa and 25-kDa peptides could represent different NDF isoforms (e.g., NDFα_2 and NDFβ_3) that have molecular weights similar to those of our protein bands or could possibly represent varying levels of glycosylation of NDF molecules. Additionally, conditioned medium from cultured neonatal OLGs also contained an immunoreactive polypeptide at 45 kDa (Fig. 2B, arrow), suggesting that NDF is secreted into the surrounding medium. All of these proteins recognized by western blotting are of OLG origin, because astrocytic contamination of our cultures was <5% and the molecular weight distribution of NDF isoforms on western blots of astrocyte lysates using antibody 1915 was different from that of OLG lysates (data not shown). In addition, incubation of membranes without primary antibody produce no visible immunoreactive polypeptides (data not shown).

We used a nested RT-PCR strategy to demonstrate the presence of mRNA for both NDFα and NDFβ isoform families in cultured neonatal OLGs (Fig. 3). First, total RNA was reverse-transcribed into cDNA, and primers designed to amplify a region of DNA common to all NDF isoforms was used to produce a 432-bp product (data not shown). This 432-bp product was used in subsequent PCR reactions using primers that would specifically amplify either NDFα or NDFβ. The second PCR amplification yielded products of 153 bp (Fig. 3, lane 2) and 144 bp (Fig. 3, lane 3), corresponding to NDFα and NDFβ, respectively. These 153-bp and 144-bp products were characterized further by sequence analysis and were determined to correspond exactly to the sequence of either NDFα or NDFβ isoform families (data not shown). An additional band at ~250 bp was also identified in the products of the

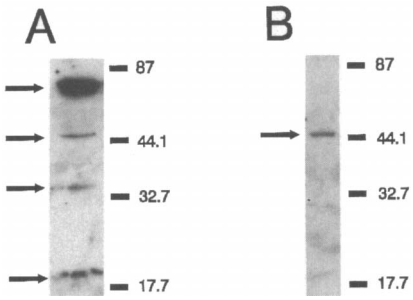

FIG. 2. Immunoblots with anti-NDF antibody 1915 from lysates of OLGs maintained in culture for 5 days (**A**) and from conditioned medium from cultured OLGs fractionated on a heparin affinity column (**B**). Lysates from neonatal OLGs maintained for 5 days contained a number of molecular mass isoforms of NDF at 80, 45, 35, and 25 kDa (A, arrows). In addition, cultured neonatal OLGs released into the medium an NDF isoform having a molecular mass of 45 kDa (B, arrow), which eluted from the heparin affinity column with 0.50–0.55 *M* NaCl. Locations of molecular mass markers (in kDa) are given as labeled bars to the right of both immunoblots. These results are representative of experiments repeated at least three times.

PCR reactions to amplify NDFβ isoforms (Fig. 3, lane 3, arrowhead). The identity of this PCR product is currently under investigation.

DISCUSSION

Reports demonstrating the presence of NRGs in OLGs have been contradictory. Vartanian et al. (1994) used in situ hybridization on slices of embryonic and postnatal rat brain, which demonstrated the presence of mRNA for ARIA (a member of the NRGβ family) in the subventricular zone, suggesting that OLG progenitors and immature OLGs contained NRGs. In this report, we confirm the presence of the NRGβ isoform family in neonatal (immature) OLGs using RT-PCR and extend these findings to demonstrate that neonatal OLGs also contain mRNA for the NRGα isoform family. These results are in contrast to those obtained by Pinkas-Kramarski et al. (1994), who found no evidence for NRGs in cultured neonatal OLGs. The discrepancy between their observations and our results is not clear at this time. Although the cultured OLGs examined in both studies are similar, the techniques used to detect NRGs are somewhat different. We performed both immunocytochemical and nested PCR techniques to detect NRGs in cultured neonatal OLGs, whereas Pinkas-Kramarski et al. (1994) first performed RT-PCR to amplify cDNA for NRGs, followed by Southern blot analysis to detect the appropriate fragment corresponding to the NRG cDNA. Pinkas-Kramarski et al. (1994) used different PCR primers from those in our studies and also did not examine NRG expression in the OLG-enriched cultures using immunocytochemistry. In addition, Pinkas-Kramarski et al. (1994) reported using immunohistochemistry to stain glial cells in the adult corpus callosum, which they correlated with glial fibrillary acidic protein staining; however, they did not examine any relationship of this glial staining with markers for OLGs. Thus, it is possible that the more sensitive nested PCR strategy and immunocytochemical techniques used in the present study may account for the differences between the two studies.

The data presented in this report raise the possibility that OLGs themselves are able to regulate their own development through autocrine/paracrine or juxtacrine (cell-to-cell) signaling using endogenous NRGs. For example, the proliferation of OLG progenitors could be regulated intrinsically by the cells themselves prior to axonal contact. Also, survival of cultured OLGs has been shown to be dependent on a glial-derived factor possibly originating from OLGs themselves (Yonezawa et al., 1996). Similar studies have shown that OLG precursors express insulin-like growth factor I, which regulates their development (Shinar and McMorris, 1995). Finally, the ability of cultured OLGs to differentiate in vitro has never been fully understood. As NRGs appear to influence OLG development, the data in this article may provide insights into the ability of these cells to extend processes without axonal stimulation.

The hypothesis that production of NRGs in OLGs is regulated developmentally is an interesting one. If different NRGs (i.e., NRGα or NRGβ) were produced and secreted at different developmental time points, different responses, such as survival, proliferation, or differentiation, could be expected. In addition, the expression of the various erbB receptor combinations

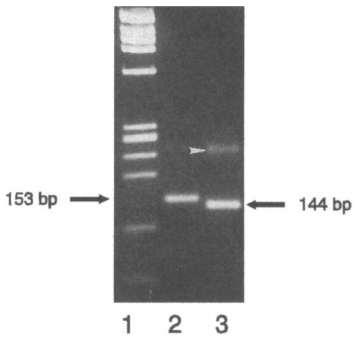

FIG. 3. RT-PCR amplification using a nested PCR strategy to detect both NDFα and NDFβ isoform families in cultured neonatal OLGs maintained for 5 days. Total RNA was reverse-transcribed and subjected to PCR using primers designed to amplify all isoforms of NDF to produce a 432-bp PCR product (data not shown). This 432-bp PCR product was used in subsequent PCR reactions with primers designed to amplify specific sequences in the epidermal growth factor-like domain to produce products of 153 bp for the NDFα isoforms (lane 2) and 144 bp for the NDFβ isoforms (lane 3). An ~250-bp product (lane 3, arrowhead) was also consistently observed in the reactions for NDFβ isoforms. Molecular weight markers (lane 1) are derived from an *Hae*III digest of ϕX-174 viral DNA (DRIgest III, Pharmacia). These results are representative of experiments repeated at least three times.

will influence the physiological response elicited by NRG stimulation. A recent report from the laboratory of Salzer has demonstrated that the erbB receptors are differentially expressed in OLG progenitors and that the expression is altered during differentiation (Canoll et al., 1996). Numerous reports have demonstrated that the formation of different homodimers and heterodimers will produce distinct biological responses (Riese et al., 1995; Beerli and Hynes, 1996; Pinkas-Kramarski et al., 1996). We are currently characterizing the NRGs produced and secreted by OLGs at different developmental stages.

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