

Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development

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SUMMARY

We mapped the distribution of neuregulin and its transmembrane precursor in developing, embryonic chick and mouse spinal cord. Neuregulin mRNA and protein were expressed in motor and sensory neurons shortly after their birth and levels steadily increased during development. Expression of the neuregulin precursor was highest in motor and sensory neuron cell bodies and axons, while soluble, released neuregulin accumulated along early motor and sensory axons, radial glia, spinal axonal tracts and neuroepithelial cells through associations with heparan sulfate proteoglycans. Neuregulin accumulation in the synaptic basal lamina of neuromuscular junctions occurred

significantly later, coincident with a reorganization of muscle extracellular matrix resulting in a relative concentration of heparan sulfate proteoglycans at endplates. These results demonstrate an early axonal presence of neuregulin and its transmembrane precursor at developing synapses and a role for heparan sulfate proteoglycans in regulating the temporal and spatial sites of soluble neuregulin accumulation during development.

Key words: Neuregulin, Spinal cord, Neuromuscular junction, Synapse, Motor neuron, ARIA, Heparan sulfate, Proteoglycan, Chick

INTRODUCTION

ARIA (acetylcholine receptor-inducing activity), a protein purified on the basis of its ability to stimulate the synthesis of acetylcholine receptors (AChRs) in embryonic myotubes, is a member of a family of growth and differentiation factors, called neuregulins (NRGs), that bind to and activate members of the EGF receptor family of tyrosine kinases *erbB2*, *erbB3* and *erbB4* (Peles and Yarden, 1993; Lemke, 1996; Burden and Yarden, 1997; Gassmann and Lemke, 1997; Fischbach and Rosen, 1997). Until recently, it was thought that all NRGs arose by alternative mRNA splicing from a single gene *NRG1* (Marchionni et al., 1993) but, within the past year, two additional genes have been discovered (Chang et al., 1997; Carraway et al., 1997; Busfield et al., 1997; Zhang et al., 1997). All NRG isoforms from the *NRG1* gene thus far examined have an EGF-like domain necessary for activation of their receptors. Most forms, like ARIA, also have an Ig-like domain that binds heparan sulfate proteoglycans (HSPGs) and leads to the deposition of these forms in the extracellular matrix (Loeb and Fischbach, 1995).

There is a growing body of evidence suggesting that ARIA, and perhaps other members of the *NRG1* family, promote the local synthesis of acetylcholine receptors at developing and mature neuromuscular synapses. *NRG1* mRNAs are concentrated in motor neurons, and the protein accumulates in motor nerve terminals at developing and mature endplates (Goodearl et al., 1995; Moscoso et al., 1995; Jo et al., 1995).

NRG receptors, *erbB2*, *erbB3* and *erbB4* have been detected by immunohistochemistry in the region of the postsynaptic membrane (Moscoso et al., 1995; Zhu et al., 1995; Altiok et al., 1995). ARIA also promotes the expression of voltage-gated sodium channels in chick muscle cells (Corfas and Fischbach, 1993), and, in mammals, it enhances expression of the AChR epsilon subunit (Martinou et al., 1991). Both effects would be expected to increase the efficacy of synaptic transmission as the target muscle fiber increases in size and the neuromuscular junction (NMJ) matures. Most significantly, mice in which one Ig-like domain of the *NRG1* allele is disrupted by homologous recombination, exhibit a 50% reduction in the density of AChRs in the postsynaptic membrane and, when challenged by low doses of curare, a reduced safety factor for neuromuscular transmission can be demonstrated (Sandrock et al., 1997). Unfortunately, homozygous mice with disruptions of the *NRG1* gene die from cardiac defects around E10 before nerve-muscle synapses form (Meyer and Birchmeier, 1995; Kramer et al., 1996). Proteins encoded by the other NRG genes have not yet been studied in detail.

For ARIA and other NRGs to act as AChR inducers at neuromuscular junctions, they must be expressed in motor neurons early in development and transported orthogradely to nerve terminals. Those isoforms that are synthesized as part of a transmembrane precursor must be cleaved and released into the synaptic cleft. NRG mRNA has been detected in embryonic chick, mouse and rat motor neurons when neuromuscular synapses are first forming (Falls et al., 1993; Orr-Urtreger et

al., 1993; Marchionni et al., 1993; Corfas et al., 1995; Goodearl et al., 1995). Little is known about the axoplasmic transport, proteolytic cleavage of transmembrane NRG isoforms, or where and how released forms accumulate. Earlier studies showed that proARIA is expressed on the surface of transfected CHO cells and that the ectodomain (ARIA) can be detected in conditioned medium (Burgess et al., 1995; Loeb et al., 1998). Activation of protein kinase C greatly enhances the release of ARIA into the medium of these transfected cells as well as cultured sensory neurons expressing endogenous NRG forms (Loeb et al., 1998). Once proteolytically cleaved and released, NRG isoforms containing an Ig-like domain adhere to the cell surface *in vivo* and can be released from the extracellular matrix by high salt, heparin or limited proteolysis (Loeb and Fischbach, 1995).

Using domain-specific antibodies, we now present evidence that the transmembrane precursor, proNRG, is expressed in developing chick embryonic motor neurons shortly after they merge from the germinal epithelium. We show that proNRG immunoreactivity is transported down motor axons as they first emerge from the neural tube. Once released from the transmembrane precursor or from other secreted isoforms, NRG immunoreactivity accumulates in the extracellular matrix within the spinal cord and at the NMJ through interactions with developmentally regulated HSPGs. These observations suggest a novel means for concentrating NRGs at specific sites within the developing central and peripheral nervous system.

MATERIALS AND METHODS

Antibodies and reagents

Mouse monoclonal IgG supernatants against Islet-1/2 (4D5) (Ericson et al., 1992) and HSPG (33-1) (Bayne et al., 1984) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa under contract N01-HD-7-3263 from the NICHD. mAb 6D2 against agrin was from Willi Halfter (University of Pittsburgh). BODIPY-BTX was from Molecular Probes (Eugene, OR). Whitehorn chick embryos were obtained from Spafas (Preston, CT), grown in an humidified, rocking incubator (Kuhl, Flemington, NJ), and staged according to Hamburger and Hamilton (1951). Timed-pregnant Swiss Webster mice were from Harland (Indianapolis, IN).

NRG antibody preparations and characterizations

Affinity-purified rabbit antisera 1310, directed against the peptide CNSFLRHARETPDSYRDS within the proximal COOH terminus of proNRG, was provided by Theresa Burgess (AMGEN). This peptide corresponds to the extreme COOH terminus of α 2C NDF and is identical to chicken proNRG amino acids 364-381, except for the presence of a G in place of the NH₂-terminal C.

An affinity-purified, polyclonal rabbit antisera against the proARIA ectodomain (183N) was prepared from a trpE fusion protein in the pATH 2 vector as described by Dieckmann and Tzagoloff (1985). The full-length 1806 bp coding sequence of proARIA (Falls et al., 1993) was subcloned into the pATH 2 vector between *Bam*HI and *Hind*III sites using primers that included a GC clamp. Fusion protein was expressed in *E. coli* RRI bacteria and subsequently isolated by preparative SDS-polyacrylamide electrophoresis (Koenig and Kunkel, 1990). Rabbit antiserum was prepared by Cocalico Biologicals, Inc. (Reamstown, PA). In order to isolate antibodies specific to the extracellular domain of proARIA, we prepared an additional trpE fusion protein containing only the first 556 nucleotides of the open reading frame using a unique *Bal*I site within the coding sequence.

Antibodies (183N) were purified by first removing any antibodies against trpE with bacterial cell lysates expressing trpE alone and subsequently using lysates expressing the trpE-556 bp insert (Koenig and Kunkel, 1990).

Both 1310 and 183N antisera were able to immunoprecipitate [³⁵S]cysteine and methionine metabolically labelled proARIA from stably transfected Chinese hamster ovary (CHO) cells as described (Loeb et al., 1998; see Fig. 2). These antisera stained CHO cells transfected with proNRG, but not untransfected CHO cells. Also, the peptide or fusion protein used to generate the antisera at 100 μ g/ml completely blocked specific E4 immunoreactivity shown in Fig. 4. While both antisera recognized proNRG on western blots of proNRG-transfected CHO cells, western blots on chick embryonic tissues produced multiple bands that were difficult to interpret perhaps due to the many alternatively spliced forms and NRG forms that were variably processed.

Mammalian, affinity-purified antiserum against the extracellular domain of proNRG (NDF) was prepared from a pooled mixture of antisera from 8 rabbits immunized with human NDF α 2₁₄₋₂₄₁. This antiserum was purified on an affinity column prepared with the above protein covalently linked to CNBr-Sepharose 4B (Sigma Chemical Co., St. Louis) according to Harlow and Lane (1988). Both the antisera and the recombinant human NDF were generous gifts from Barry Ratzkin at AMGEN.

Immunohistochemistry

20 μ m cryosections on Superfrost Plus glass slides (Fisher) were prepared from staged chick embryos and timed-pregnant mouse embryos that had been fixed in 4% paraformaldehyde in PBS for 1 or more hours at room temperature. Antibodies were applied overnight at 4°C in a humidified chamber in 100 mM sodium phosphate (pH 7.5), 0.5 M NaCl, 0.5% Triton X-100 and 10% heat-inactivated normal goat serum at the following dilutions: 1310 1:300; 183N 1:100, Isl-1/2 1:10; 33-1 1:10, and NDF at 1:50. Slides were washed twice for 5 minutes each in 40 ml PBS, then incubated in secondary antibodies in the same buffer for 1 hour at room temperature, followed by two washes in PBS and mounted with 5% propylgallate. 183N immunoreactivity was easily seen using a Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) at 1:500. Since the 1310 immunoreactivity was barely detectable with the Cy3 secondary, we amplified the signal using the TSA-direct kit from NEN Life Sciences (Boston, MA) according to the manufacturer. Goat anti-mouse IgG coupled to Cy2 (1:500) was from Amersham Life Sciences (Arlington Heights, IL) and used for double-labeling experiments with RT97 and 33-1.

Immunofluorescent photomicrographs were taken with a Nikon Eclipse 800 or Microphot microscope equipped with Cy-3 and fluorescein filter sets. Photographs were taken directly or digital images were obtained using a Photometrics, cooled CCD camera (Tucson, AZ).

In situ hybridization

20 μ m transverse sections through chick brachial level spinal cord were prepared as described above. *In situ* hybridization was performed using both antisense and sense ³⁵S-labelled RNA probes corresponding to the full-length proARIA-1, 2.3 kB sequence as described previously (Falls et al., 1993; Corfas et al., 1995). After hybridization, slides were washed, exposed to NTB-2 Kodak emulsion for 7 days and photographed using a dark-field illuminator mounted on a Nikon Eclipse 800 microscope.

In order to map the distribution of NRG isoforms containing the Ig-like domain, we prepared a 177 bp probe corresponding to base pairs 137-313 of the open reading frame of proARIA-1 using paired PCR primers: TGC TAA GGT GTG AAA CCA CTT CAG and ATG CGT ATT CCC CAG CGT CAG. This fragment was subcloned into the vector pCR 2.1 (Invitrogen) from which *in situ* probes were made as above.

Release of NRG from the ECM

Adjacent serial sections from stage 24 chicken embryos were covered with parafilm and incubated in a humidified chamber at 37°C for 90 minutes with PBS/0.1% BSA alone, PBS/0.1% BSA with 0.85 M NaCl (1 M NaCl final), or PBS/0.1% BSA containing 0.04 Units/ml Heparitinase (Segeiaku, Japan). Degradation of HSPGs from fixed tissue has been described previously (Kogaya et al., 1990). The slides were washed for 5 minutes twice in PBS at room temperature and stained with 183N or 1310 as described except a solution of PBS (pH 7.4), 0.3% Triton X-100, 10% heat-inactivated goat serum was used for primary and secondary antibodies.

Electron microscopy: ultrathin cryosections

Anterior latissimus dorsi (ALD) muscles from E18 chick embryos were immersion fixed in freshly prepared 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 hour. The muscles were then washed in 0.1 M phosphate buffer, cut into small blocks and cryoprotected in 15% polyvinylpyrrolidone in 2 M sucrose for a minimum of 2 hours before being frozen in liquid nitrogen (Tokuyasu, 1989). Ultrathin 60-70 nm sections were cut on a Reichert ultramicrotome equipped with a cryochamber. The sections were picked up on 3:1 sucrose:methyl cellulose droplets (Liou et al., 1996) and transferred to carbon-coated 200 mesh grids. Immunolabelling was performed according to the procedure of Slot and Geuze (1985). 0.1% BSA was used as the blocking buffer. The primary antibodies were diluted 1:40 (183N) and 1:100 (33-1) with 1% BSA in PBS. Secondary antibodies used were protein A labelled with 10 nm gold for 183N diluted 1:40 and 10 nm gold-labelled goat anti-mouse IgG (Nanoprobes, Inc., Stony Brook, NY) diluted 1:40 for 33-1. After labelling, the sections were fixed in 2% glutaraldehyde and stained with 2% neutral uranyl acetate followed by 10 minutes in 1.8% methylcellulose and 0.2% uranyl acetate. Grids were allowed to dry before being examined in a JEOL 100CX or JEOL 1200 electron microscope. Histograms of the distance of gold particles from the muscle membrane were calculated from digitized, calibrated photomicrographs from several different NMJs.

RESULTS

proNRG is expressed in postmigratory motor neurons and is transported down their axons

Using a full-length chicken proARIA probe, we examined the distribution of NRG mRNA in the developing chick spinal cord by *in situ* hybridization. Adjacent transverse sections cut through the brachial spinal cord were examined in stage 16-24 embryos (Hamburger and Hamilton, 1951), approximately E2-E4 (Fig. 1). All stages were hybridized with the same probe and the emulsion was exposed for the same time and developed in the same way so that the grain density reflected the relative amount of mRNA. The signal in the ventrolateral cord was clearly above background at stage 16, the earliest time examined. The signal increased over the next 2 days (through stage 24). By this stage, a majority of motor neurons have been born and had migrated to their final positions in the ventral horn (Hamburger, 1948).

NRG mRNA was also evident in the dorsal portion of the spinal cord lateral to the germinal zone (Fig. 1, stages 20, 24). The dorsal horn grain density was far below that in the ventral horn at all ages examined, but it was clearly greater than control sections hybridized with sense strand cRNA. Though not examined further here, NRG mRNA was present outside of the spinal cord early in development in dorsal root ganglia (DRG), autonomic ganglia and dermamyotome (Fig. 1).

A dorsal-ventral gradient was evident within the dermamyotome.

The full-length probe used for these experiments should recognize all known *NRG1* splice variants (see Fischbach and Rosen, 1997 for review). We were particularly interested in NRG isoforms, like ARIA, containing an Ig-like domain because of the likely role of this domain in localizing released NRG forms within the extracellular matrix (Loeb and Fischbach, 1995) and in promoting synaptic efficacy *in vivo* (Sandrock et al., 1997). Therefore, we prepared a smaller probe restricted to the Ig-like domain to examine the distribution these isoforms (see Fig. 2A). This probe hybridized in the same pattern as we observed with the full-length probe. In stage 24 chick cords, expression was maximal in the ventral horn, dorsal

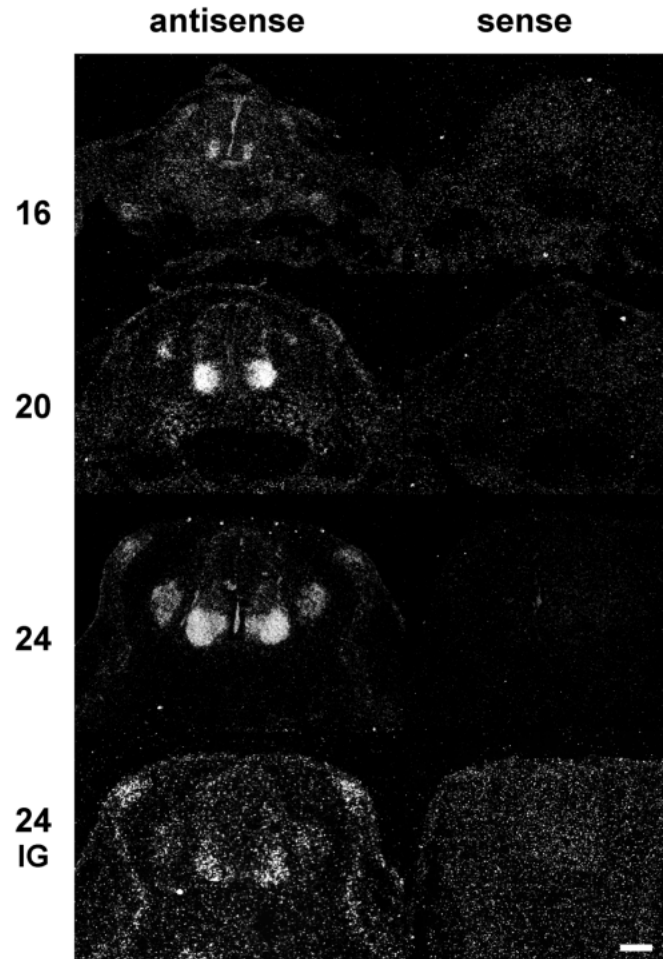
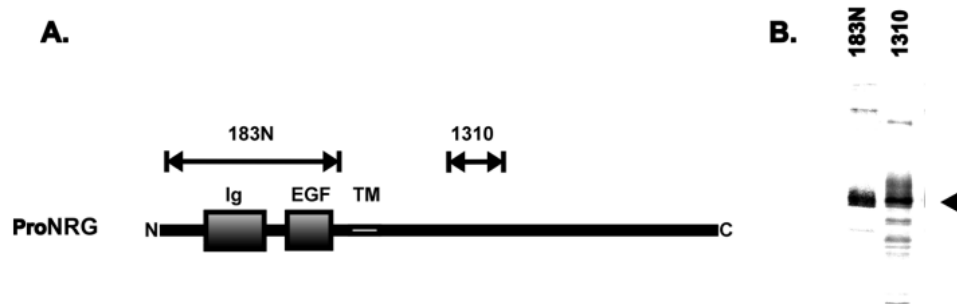


Fig. 1. Developmental expression of NRG mRNA in embryonic chick spinal cord. Adjacent transverse sections through stage 16 through 24 brachial level embryonic chick spinal cord were hybridized with a full-length, ³⁵S-labelled proNRG probe (antisense) or a sense control probe (sense). While NRG mRNA was highly expressed in the ventral horn (VH) overlying motor neurons, there was additional message in dorsal root ganglia (DRG), dorsal dermamyotome (DM), and at lower levels throughout the dorsolateral spinal cord. An adjacent section at stage 24 was hybridized with a probe specific to neuregulin isoforms containing the Ig-like domain (24 IG). Using this probe, the same general pattern was seen, but of lower intensity likely related to the smaller probe length. (Bar, 100 μm).

Fig. 2. Summary of proNRG domain structure and specificity of antisera.

(A) ProNRG is the transmembrane precursor of NRG. The secreted ectodomain (NRG) contains an EGF-like domain (EGF) that binds to the *erbB* family of tyrosine kinase receptors and an Ig-like domain (Ig) that binds to heparin and is responsible for extracellular matrix interactions. ProNRG has a transmembrane domain (TM) and a long COOH-terminal cytoplasmic domain.

183N and 1310 identify the epitopes used to generate domain-specific antiserum against the ectodomain and cytoplasmic domain, respectively. (B) Specificity of the 183N and 1310 affinity-purified polyclonal antisera was determined in part by their ability to immunoprecipitate ³⁵S-labelled proNRG (80-90 kDa) in stably transfected CHO cells (indicated by arrow). Lower molecular weight bands reflecting the cytoplasmic domain that remains cell-associated after proteolytic release of NRG are immunoprecipitated only by the 1310 antisera.



root ganglia and the dorsal portion of the dermamyotome (Fig. 1, bottom). Because the probe length was considerably smaller, it was not possible to compare the proportion of Ig-containing NRG messages; however, the identical pattern of expression suggests that Ig-domain-containing NRG isoforms are present in all areas of NRG expression, including motor neurons, at a time prior to neuromuscular synapse formation.

We also examined the distribution of NRG-like immunoreactivity during early developmental stages with an affinity-purified antiserum called 1310 raised against a peptide in the cytoplasmic domain (Burgess et al., 1995; Fig. 2A). This epitope is located close to the transmembrane segment, in a region that is common to all known transmembrane isoforms. The specificity of 1310 was demonstrated both by its ability to immunoprecipitate recombinant, metabolically labelled chicken proARIA in stably transfected fibroblasts (Fig. 2B), and by reduction in fluorescence when 1310 was added along with the peptide epitope. The 1310 immunoreactivity was relatively weak and therefore was routinely amplified using a method where tyramide-coupled fluorochromes are deposited at sites of primary antibody deposition (Bobrow et al., 1989).

Transverse sections of brachial-level, stage 13-24 spinal cords were double labelled with 1310 and the early motor neuron transcription factors *islet-1* and *islet-2* (*isl-1/2*) (Tsuchida et al., 1994; Fig. 3). At stage 13, prior to motor neuron birth, no 1310 or *isl-1/2* immunoreactivities were detected within the neural tube. ProNRG immunoreactivity first appeared at stage 16 in the ventrolateral spinal cord in newly born motor neurons. The intensity of the NRG signal increased as the number of motor neurons increased through stage 24. This time course matches that seen by *in situ* hybridization in Fig. 1.

At these early stages, the proNRG and *isl-1/2* immunoreactivity did not overlap precisely. At stage 16, the more medial, *isl-1/2*-positive motor neurons expressed little proNRG immunoreactivity, whereas higher levels were expressed in motor neurons that had migrated to the lateral margin of the ventral horn. This produced a gradient of proNRG expression from lateral to medial as shown at higher magnification in Fig. 3B. By stage 24, proNRG labelling filled in more medially and was more uniform throughout the ventral horn. This lag between the expression of *islet-1/2* and NRG raises the possibility that NRG expression in motor neurons is

not regulated solely by an *islet-1/2*-triggered developmental program. Extrinsic factors may also be needed.

ProNRG (1310) immunoreactivity was evident in motor axons from the moment that they emerged from the neural tube to invade surrounding mesodermal tissues (Fig. 3), and extended throughout the entire length of axons including growth cones as they entered the limb bud to contact developing muscle masses at stage 24 (Fig. 4A). The presence of the proNRG precursor in motor neuron growth cones places it in an ideal position to act during the initial stages of nerve-muscle synapse formation.

Released NRG accumulates in the spinal cord through interactions with HSPGs

In order to determine the distribution of NRGs proteolytically released from the transmembrane precursor, we prepared an affinity-purified antiserum called 183N directed against the entire NH₂-terminal, extracellular domain of proARIA (Fig. 2A). Specificity of 183N was demonstrated by selective immunoprecipitation of metabolically labelled proARIA (Fig. 2B) and by blocking immunoreactivity with recombinant fusion protein (Fig. 4A, right). Signal amplification was not needed to visualize staining with 183N, perhaps suggesting a greater abundance of this antigen.

At stage 24, both 1310 and 183N intensely labelled motor and sensory axons as they invaded the limb bud bifurcating to innervate the dorsal and ventral muscle masses (Fig. 4A). Despite this strong correspondence within the peripheral nerve, a strikingly different pattern of immunoreactivity between 1310 and 183N was observed within the spinal cord. Whereas 1310 immunoreactivity was intense in motor neuron cell bodies, 183N immunoreactivity was low over the motor neuron cell bodies, but was intense over axonal tracts and diffusely stained many other structures throughout the spinal cord.

Fig. 4B highlights some of these differences in E5 chick spinal cords labelled with 1310 or 183N. In order to demonstrate axonal labelling, comparison was made with an antibody against neurofilament proteins (RT97). ProNRG (1310) immunoreactivity was restricted to motor and sensory neuron cell bodies and their axons forming the ventral root, the dorsal root and the dorsal root entry zone. The neurofilament antibody staining colocalized with the motor and sensory neurons and their axons (filled arrows). The 183N antisera also stained motor and sensory neuron axons. However, additional

labelled structures included commissural axons, radial glia, neuroepithelia, and ascending and descending axonal tracts along the outer margin of the cord, that colocalized with neurofilament staining (open arrows).

The strikingly different expression pattern seen between cytoplasmic (1310) and the extracellular (183N) epitopes of proNRG within the chick spinal cord was also seen in embryonic mouse spinal cord (Fig. 4C). In the E12 mouse spinal cord, 1310 stained motor and sensory neurons and their axons, identical to that seen in the chicken. While the 1310 antisera recognized both mammalian and avian NRGs, 183N did not cross-react with mammalian tissues. We therefore used an affinity-purified antisera (NDF) against the extracellular domain of human NRG. Although this antisera did not cross-react with chicken tissues, it produced an identical staining pattern in E12 mouse spinal cord to that seen in E5 chick spinal cord using 183N.

One explanation for the difference in staining pattern observed for antibodies against the transmembrane precursor and the extracellular domain is that, once proteolytically processed and released, soluble NRG diffuses away and adheres to specific extracellular sites. To test this, we examined the stability of the 183N immunoreactivity on sections of E4 chicken spinal cord after treatments designed to disrupt interactions between soluble NRG and the extracellular matrix (Fig. 5, top). Either high salt treatment, which disrupts ionic interactions, or heparitinase treatment, which specifically degrades HSPGs, resulted in a marked reduction of 183N immunoreactivity in all of the labelled structures. This suggests that most of the 183N immunoreactivity was indeed associated with the extracellular matrix. As a control, identically treated sections stained with 1310 had no reduction in staining intensity as would be expected for a membrane-anchored protein (Fig. 5, bottom). The high salt treatment was consistently more effective than heparitinase suggesting either that the enzymatic digestion of HSPGs was not complete or that other negatively charged matrix components were also binding NRG. However, treatment of a similar section with neuraminidase, which removes negatively charged sialic acid residues such as on PSA-NCAM, caused no reduction in 183N signal intensity over all of these regions (data not shown).

Matrix-bound NRG is expressed at lower levels later in development

Using the 183N antisera, we compared the distribution and signal intensity of matrix-bound NRG in chick spinal cord sections processed in parallel at E5 and E11 (Fig.

6A). At E11, the 183N immunoreactivity was dramatically reduced, but not totally absent, within the spinal cord and nerve roots. Using higher magnification and longer exposure times at E18, 183N immunoreactivity could be detected in the matrix surrounding motor neuron cell bodies in grey matter and surrounding glia and axons in the developing white matter (Fig. 6B). In contrast, 1310 immunoreactivity remained high within the motor neuron cell bodies and proximal processes. As seen at earlier stages, this inverse pattern of staining between 1310 and 183N suggests that a majority of released NRG isoforms continue to accumulate outside motor neurons in the extracellular matrix.

Coincident accumulation of NRG and HSPGs at NMJs

Despite the early presence of NRG immunoreactivity in motor neuron axons from E2 onward, we have been unable to detect NRG immunoreactivity at NMJs until after E15 (Goodearl et

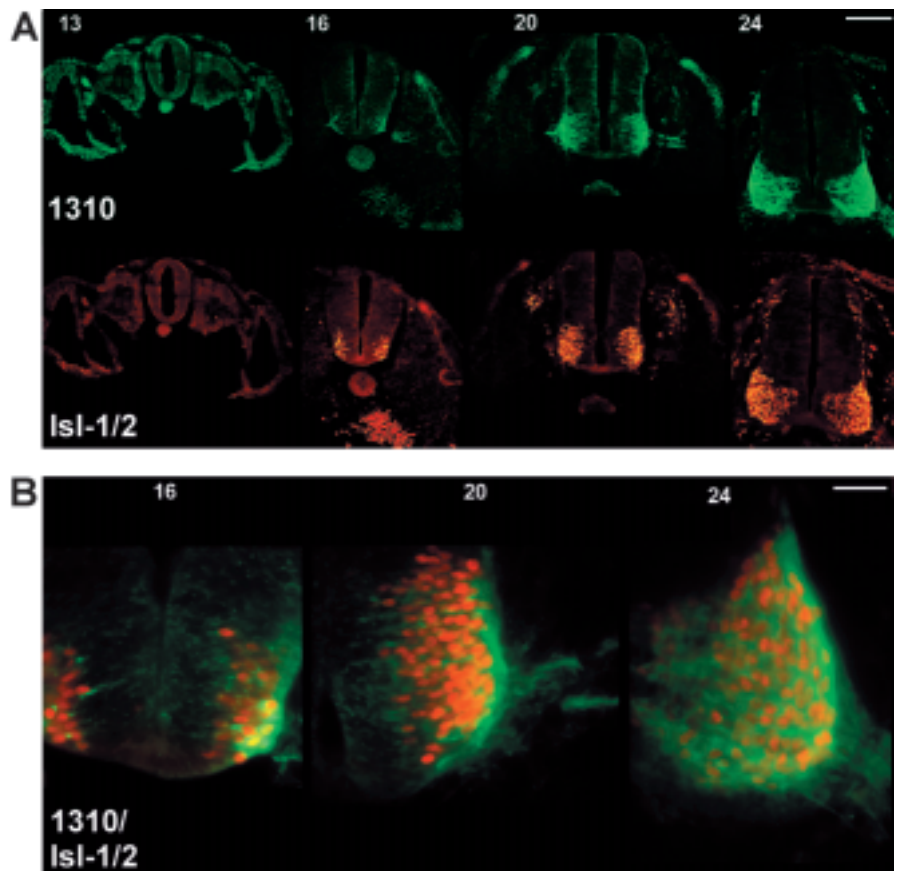


Fig. 3. ProNRG immunoreactivity appears in postmigratory motor neurons and their axons. (A) Transverse sections through stage 13–24 embryonic chick brachial spinal cords were double labelled with 1310 and *isl-1/2*. Prior to motor neuron birth at stage 13, no labelling was detected. The appearance of proNRG immunoreactivity in the ventrolateral neural tube occurred coincident with the expression of motor neurons expressing *islet-1/2* at stage 16 and increased through stage 24. ProNRG immunoreactivity was also detected in motor neuron axons as they first emerged from the neural tube. (B) At higher magnification, fused images at stages 16 and 20 revealed that more medial, migrating motor neurons expressed *isl-1/2* immunoreactivity (red), but very little proNRG immunoreactivity (1310, green). In contrast, those motor neurons that had completed their migration expressed high proNRG levels. This gradient was no longer apparent by stage 24. (Bar, 100 μ m for A and 30 μ m for B.)

al., 1995). We have re-examined this using 183N on ALD muscles from E14-E18 chick embryos. At E14, NRG immunoreactivity was diffusely distributed along muscle fibers (Fig. 7, top panel) and highly expressed in intramuscular nerves (not shown). At E16, NRG immunoreactivity first became detectable at synapses with a corresponding reduction in extrasynaptic NRG immunoreactivity. By E18, NRG immunoreactivity was highly concentrated at synapses with markedly reduced levels at extrasynaptic locations.

Because of their association with NRG in the developing spinal cord, we hypothesized that synaptic HSPGs may function to concentrate NRG at NMJs. HSPGs are known to be concentrated in the basal lamina of NMJs *in vivo* and to become concentrated in the basal lamina of NMJs *in vitro* coincident with clusters of AChRs (Anderson and Fambrough, 1983; Anderson et al., 1984; Bayne et al., 1984; Chiu and Sanes, 1984). When we labelled ALD muscles with both 183N and monoclonal antibody 33-1 directed against a chicken, synapse-associated HSPG (Bayne et al., 1984), we found a striking correspondence (Fig. 7, second panel). The HSPG immunoreactivity was diffusely distributed along muscle fibers at E14 and became progressively concentrated at NMJs together with NRG. By E18, we saw a steep gradient between junctional and extrajunctional immunoreactivity for both antigens.

Agrin is a large HSPG synthesized both by motor neurons and muscles (Tsen et al., 1995; Bowe and Fallon, 1995). Using monoclonal antibody 6D2 against agrin, we found a similar developmental redistribution of agrin from E14-E18. Between E16 and E18, there was a marked reduction of immunoreactivity at extrajunctional sites with relative preservation at synapses identified by double labeling with α -bungarotoxin (α -BTX) (Fig. 7, bottom two panels). The change in the expression of these two HSPGs from extrajunctional to junctional sites, therefore, may be a developmentally driven force that functions to concentrate NRGs at neuromuscular synapses.

We also examined the distribution of NRG (183N) and HSPG (33-1) immunoreactivity within the E18 ALD NMJs by immunogold electron microscopy. NRG immunoreactivity was highly concentrated along the lamina densa of the basal lamina adjacent to the nerve terminal (Fig. 8A). The HSPG antibody was restricted to an adjacent

region within the basal lamina and lamina lucida closer to the muscle membrane (Fig. 8B). A histogram of the distances of immunogold particles from the muscle membrane

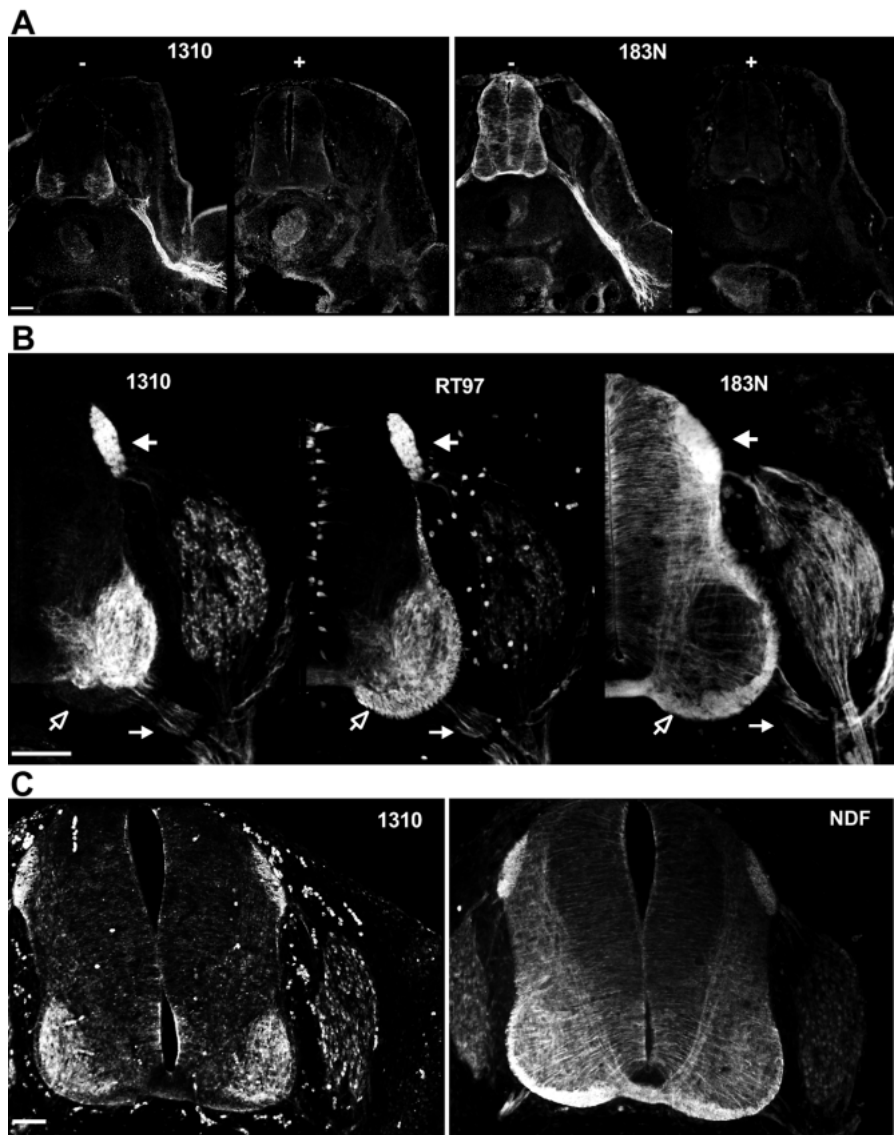


Fig. 4. Antibodies against the cytoplasmic and extracellular domains of proNRG produce different staining patterns within the spinal cord. (A) Transverse sections through brachial spinal cord and peripheral nerve entering the limb bud at stage 24 (E4) were stained with 1310 antisera in the absence (–) or presence (+) of the peptide used to generate the antiserum. A similar set of transverse sections stained with 183N antisera in the absence (–) or presence (+) of competing fusion protein demonstrated similar labelling over the entire length of the motor and sensory axons, but a more diffuse pattern in the spinal cord. (B) E5 spinal cords with associated DRG and nerve roots were double labelled with 1310 and RT97 against neurofilament protein. Motor and sensory neuron cell bodies and their axons were intensely labelled with both 1310 and RT97 as was the ventral root (small white arrows) and sensory axons in the dorsal root entry zone (large white arrows). An adjacent E5 section stained with 183N also showed intense labelling of motor and sensory neuron axons in the ventral root, dorsal root, and dorsal root entry zone. Additional labelling was noted in the neuroepithelium, commissural axons, radial glia, and ascending and descending spinal tracts that were also labelled with the neurofilament antibody (open arrows). (C) The pattern of both COOH- and NH₂-terminal NRG immunoreactivity was assessed in the 12 day-old embryonic mouse spinal cord. Staining with 1310 revealed an identical pattern seen with this antisera on chick tissue, staining motor and sensory neurons and their axons. Using an antiserum that recognizes the extracellular domain of a mammalian neuregulins (NDF), an identical pattern was seen as was observed in the chick using 183N. (Bars, 100 μ m.)

demonstrates the clearly separate, but adjacent locations of these two epitopes within the basal lamina (Fig. 8C). The negatively charged glycosaminoglycan side-chains of HSPGs have been shown to be present in the outer lamina lucida of the muscle basal lamina (Kogaya et al., 1990), suggesting that the protein core of the HSPG is within the lamina densa where most of the immunogold grains were localized. The localization of NRG immunoreactivity to the margin between the outer lamina densa and the lamina lucida, therefore, might be expected because of interactions between NRG and these negatively charged glycosaminoglycans.

DISCUSSION

Early expression of NRG and its transmembrane precursor in motor neurons and their axons

We have shown here that NRG mRNA and protein are expressed in motor neurons shortly after their birth and migration from the germinal epithelium. The initial expression of NRG in motor neurons lags behind the expression of the motor neuron transcription factors *islet-1* and *islet-2*. Only the *isl-1/2*-positive cells that had completed their migration to the lateral wall of the neural tube expressed detectable NRG levels producing a lateral-to-medial gradient of NRG expression. More lateral motor neurons are older and the first to extend their axons into surrounding mesoderm (Ericson et al., 1992). It has recently been shown that disruption of the mouse *islet-1* gene blocks NRG expression in ventral spinal cord (Plaff et al., 1996), suggesting that the expression of *islet-1* is necessary for NRG expression in motor neurons. It may be that *islet-1* is also sufficient, but with a long lag in NRG expression following *islet-1*. Alternatively, the *islet-1* and *islet-2* transcription factors may not be sufficient. Extrinsic factors either from the lateral neural tube or from surrounding mesodermal tissues may be needed to induce NRG expression. It is interesting, in this regard, that neurotrophic factors expressed in embryonic muscle rapidly and selectively increase NRG expression in cultured rat motor neurons (Loeb and Fischbach, 1997).

We found that proNRG was present all along motor and sensory axons from the moment that they formed. The uncleaved, transmembrane NRG precursor may interact directly with erbB receptors in muscle or glia. Such 'juxtacrine' signaling between two adjacent cells has been shown to occur with other transmembrane precursors of soluble factors (Massague and Pandiella, 1993).

These results also raise the possibility that soluble forms of NRG may be generated and released from proNRG along axons and at their terminals. Extracellular signals that activate protein kinase C lead to a rapid release of ARIA from proARIA in transfected fibroblasts and cultured sensory neurons (Loeb et al., 1998). Thus, once transported down an axon, signals from nearby cells may regulate NRG release through post-translational processing

mechanisms and provide a dynamic means to control the amount of NRG present at specific sites during development.

NRG splice-variants containing the Ig-like domain are expressed in motor neurons during synapse formation

All NRGs contain an EGF-like domain that is necessary for binding and activation of the erbB receptors. Some isoforms, like ARIA, also contain an NH₂-terminal Ig-like domain that appears to be important for binding released NRG forms to the extracellular matrix (Loeb and Fischbach, 1995). Our *in situ* hybridization experiments showed that mRNA encoding this Ig-like domain was distributed in an identical pattern to that detected with a probe recognizing all NRG isoforms. This was observed previously in E5 and E21 chick motor neurons with a slightly longer probe containing additional NH₂-terminal sequences (Goodearl et al., 1995). Thus, although Ig-containing forms of NRG mRNA are not the most abundant forms in spinal cord (Corfas et al., 1995; Meyer et al., 1997; Yang et al., 1998), they are present in motor neurons and expressed very early in development. Two other studies failed to detect mRNA for Ig-containing isoforms in newly born motor neurons (Meyer et al., 1997; Yang et al., 1998). We cannot account for their negative results. They may reflect technical or species differences.

NRG splice variants have also been identified in which a cysteine-rich domain (CRD) is substituted for the Ig-like domain. These forms have been found to be highly expressed in early motor and sensory neurons (Ho et al., 1995; Meyer et al., 1997; Yang et al., 1998). The 1310 antisera used in the

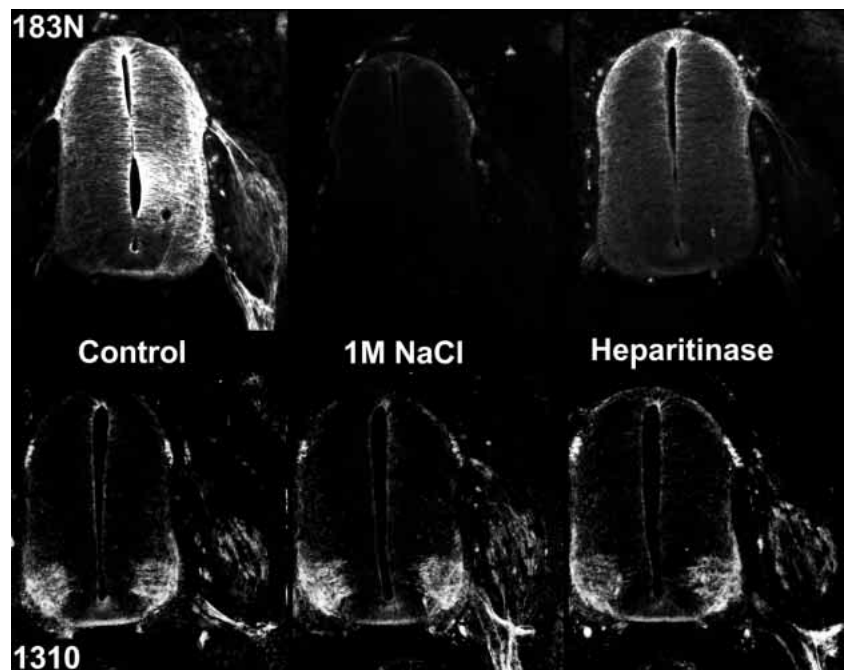


Fig. 5. A majority of the NH₂-terminal immunoreactivity can be released by high salt or HSPG degradation. Adjacent transverse spinal cord sections of chick E4 embryos were treated with PBS alone (control), 1 M NaCl in PBS, or the HSPG-degrading enzyme Heparitinase in PBS for 1 hour at 37°C. Sections were subsequently equilibrated in PBS and then stained with either 183N (top) or 1310 (bottom). While both the 1 M NaCl and Heparitinase treatment resulted in a significant reduction in 183N immunoreactivity, there was no reduction in the 1310 immunoreactivity.

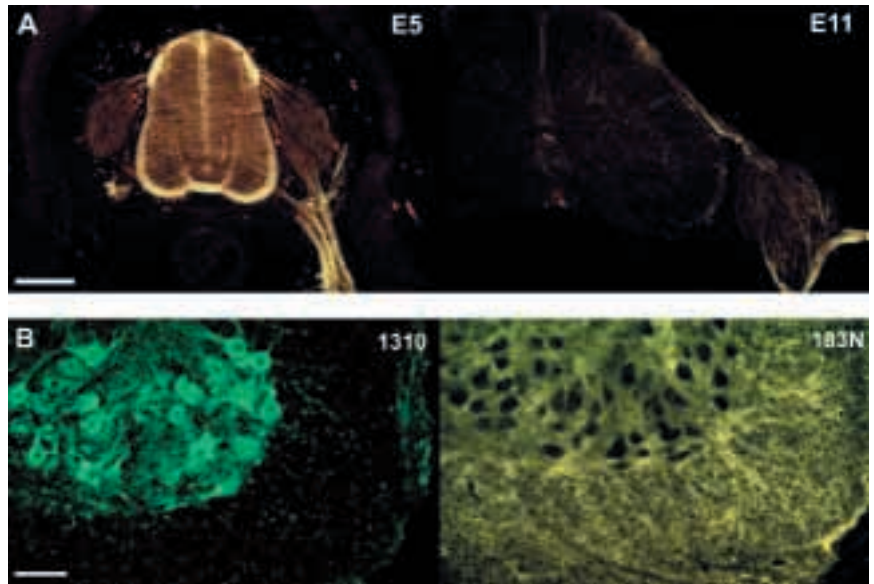


Fig. 6. Matrix-bound NRG is present at reduced levels after E5. (A) Comparison was made of the distribution and staining intensity of NRG with 183N antisera in transverse, brachial sections of chick embryonic spinal cord at E5 and E11. By E11, most of the immunoreactivity was reduced. (B) At E18, a higher-magnified, longer exposure of the ventrolateral spinal cord demonstrated that 183N immunoreactivity was still present in the matrix surrounding motor neuron cell bodies in the grey matter and in the developing white matter. In contrast, 1310 immunoreactivity in a nearby section remained expressed at high levels within the cell bodies and proximal processes of motor neurons. (Bar = 100 μm top, 75 μm bottom.)

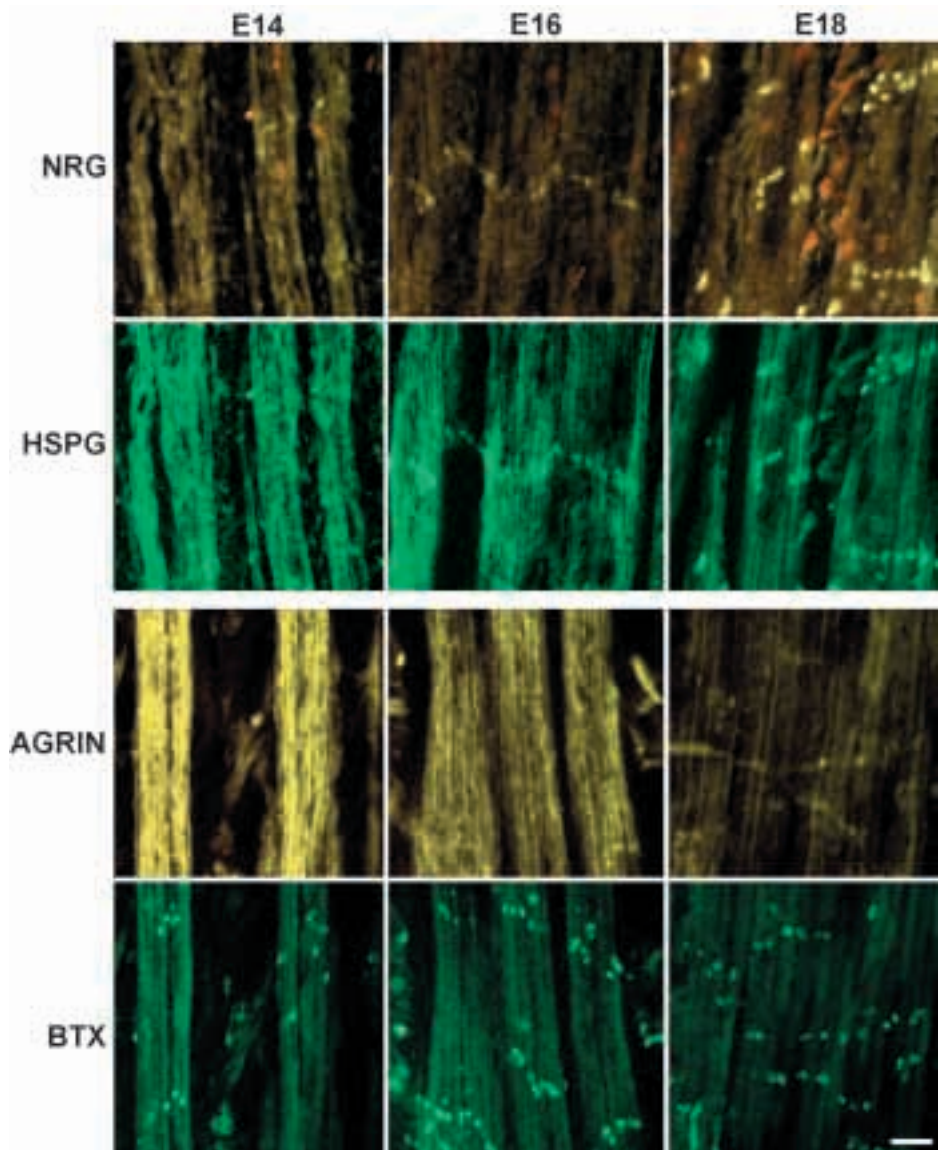


Fig. 7. NRG accumulates at NMJs coincident with HSPGs. Chick ALD muscles at E14, E16 and E18 were double labelled with 183N and a muscle-derived HSPG (33-1) (upper two panels). Both antigens were diffusely distributed along muscle fibers seen at E14 and progressively became concentrated at NMJs by E18 with a corresponding reduction in extrasynaptic immunoreactivity. Agrin immunoreactivity (6D2) also became concentrated at synaptic sites, double labelled with BODIPY- α -bungarotoxin (lower two panels). (Bar, 30 μm .)

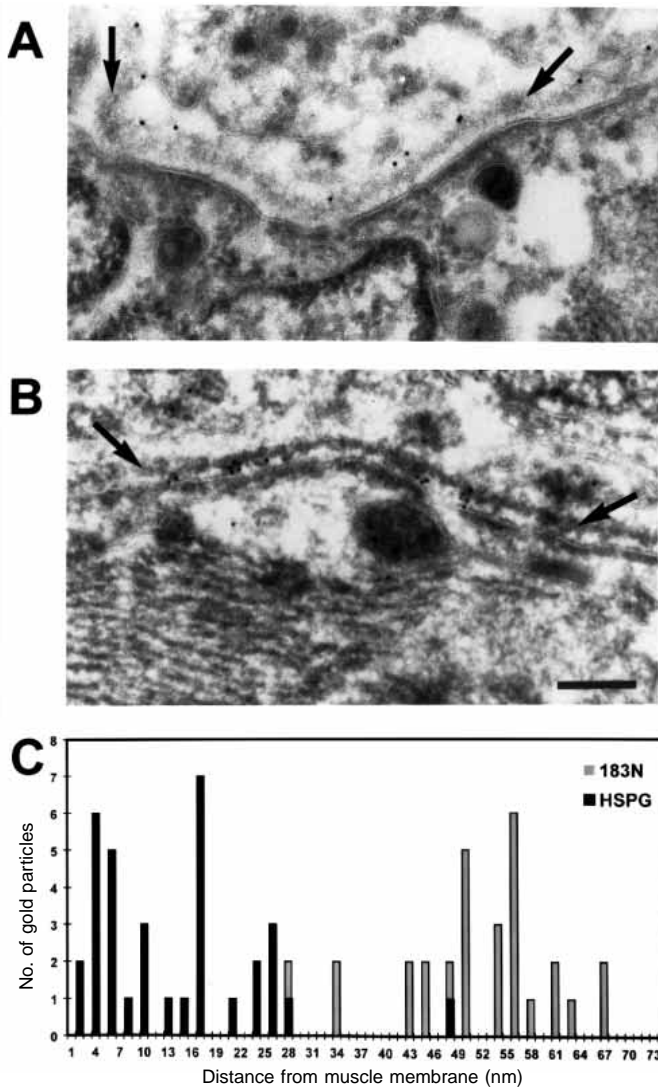


Fig. 8. NRG and HSPG immunoreactivities localize to adjacent regions of the synaptic basal lamina. (A) Immunogold labelling of an E18 ALD NMJ with 183N was restricted to the outer portion of the lamina densa of the basal lamina adjacent to the nerve terminal membrane. (B) The HSPG antibody (33-1) labelled the lamina lucida adjacent to the muscle membrane and extended into the lamina densa of the basal lamina. Arrows in A and B mark the lamina densa within synaptic basal lamina. (Bar, 0.2 μ m) (C) This histogram, prepared from multiple electron micrographs, measured the distance in nm of gold particles to the postsynaptic muscle membrane for HSPG and NRG (183N).

present study detects all NRGs with a cytoplasmic domain, regardless of whether they have an Ig-like or CRD domain. Both 183N and the pooled mammalian antisera (NDF) were prepared using Ig-containing, extracellular domains. Given that a majority of 183N immunoreactivity is removed by high salt treatment or heparan sulfate degradation, we suspect that most of this immunoreactivity represents NRG isoforms with the Ig-like domain. However, we cannot rule out that these antisera also react with NRG isoforms lacking this Ig-like domain. To date, there is no evidence that CRD-containing isoforms bind to HSPGs or associate with the extracellular matrix.

Accumulation of NRG at sites of HSPG deposition suggests novel sites of NRG action

While a majority of proNRG message and protein was localized to motor and sensory neuron cell bodies and axons, our chicken and mouse antisera against the NH₂-terminal, extracellular domain stained additional structures including axonal tracts, radial glia and neuroepithelia. Most of this immunoreactivity was released by high salt or an enzyme that specifically degrades heparan sulfate, suggesting that a majority of this immunoreactivity reflects NRG associated with HSPGs. This finding is consistent with previous observations where NRG protein and tyrosine phosphorylation activity was released from the surface of freshly dissociated chick spinal cord by high salt, heparin or selective proteolytic cleavage of the Ig-like domain (Loeb and Fischbach, 1995).

While NRG immunoreactivity associated with the extracellular matrix was very strong, we saw little staining of motor and sensory neuron cell bodies with the 183N (chick) and NDF (mammalian) antiserum. We suspect that this is due to the release of soluble NRGs into the extracellular media resulting in a depletion of the extracellular domain within cell bodies. Once expressed on the cell surface, proNRG is rapidly cleaved to release the soluble ectodomain in transfected fibroblasts (Loeb et al., 1998). Therefore, much of the 1310 immunoreactivity seen in cell bodies may reflect the 'stump' or remaining cytoplasmic domain associated with the cell after proteolytic cleavage. An alternative explanation is that the 183N and NDF antisera are recognizing NRG forms that are synthesized without the cytoplasmic and membrane-spanning sequences (β 3 forms; Marchionni et al., 1993).

The exact role of matrix-bound NRG in the developing spinal cord is not yet clear. HSPGs have a unique role of acting as reservoirs for many other soluble growth and differentiation factors. In fact, the distribution of the heparin-binding guidance factor netrin-1 shows similarities to that of NRG in the embryonic spinal cord (T. E. Kennedy and M. Tessier-Lavigne, personal communication). NRG is thus positioned to participate in the development of early ascending, descending and commissural axons. NRGs may also interact with central and peripheral glia, known to respond to NRG for their propagation, differentiation and survival (for review, see Lemke, 1996; Trachtenberg and Thompson, 1996). Staining along radial glia supports recent findings documenting an important new role for the NRGs in cell migration along radial glia (Rio et al., 1997; Anton et al., 1997). Additional roles have been proposed for NRGs in cell fate determination of neural crest cells (Shah et al., 1994), suggesting possible effects of NRGs in the developing neuroepithelia. Later in development, as the peripheral nerves and axonal tracts within the spinal cord mature, the staining intensity decreases dramatically suggesting that many of the roles that NRG plays here may be transient.

HSPGs have been identified and localized within the developing central and peripheral nervous systems. For some, the expression patterns clearly parallel that seen here for NRG making them good candidates for NRG-binding proteoglycans. One of these is cerebroglycan, localized in the developing embryo along motor and sensory axons, spinal axonal tracts and commissural axons (Ivins et al., 1997). Similar to what we observed here for NRG, little cerebroglycan immunoreactivity was present in motor neuron cell bodies and cerebroglycan

immunoreactivity along axonal tracts became significantly reduced as these tracts matured. Agrin is another HSPG recently shown to be present in central and peripheral axonal tracts of the developing nervous system (Tsen et al., 1995; Halfter et al., 1997) and may also participate in localizing soluble NRG. It seems likely HSPGs expressed at specific times during nervous system development may direct the localization of Ig-containing NRG forms to specific regions.

NRG accumulation at maturing synapses may be driven by a change in the distribution of HSPGs

NRG immunoreactivity at NMJs becomes obvious relatively late in development: on E16 in the chick and around birth in mammals (Goodearl et al., 1995; Sandrock et al., 1995; Jo et al., 1995; Moscoso et al., 1995). The present study confirms these observations from our earlier work in the chick using different antibodies (Goodearl et al., 1995). This is puzzling given that NRG is expressed soon after motor neurons are born and carried to the distal ends of growing motor axons at the time of initial nerve-muscle (and nerve-glia) contact. We suggest that soluble NRG is released from nerve endings from E2 onward; however, the dramatic rise in NRG immunoreactivity at NMJs between E16 and E18 results from a relative concentration of HSPGs in the synaptic basal lamina. Consistently, we observed a dramatic change in the distribution of a muscle-derived HSPG (33-1) and agrin (6D2) in chick ALD muscle from E14-E18. The pattern of these proteoglycans changed from a diffuse distribution along the entire muscle length to a highly restricted pattern of expression at endplates. A different anti-agrin antibody (5B1) (Fallon and Gelfman, 1989) revealed a similar reduction in extrasynaptic agrin immunoreactivity with a corresponding increase at junctional sites over this same period.

NRG immunoreactivity became progressively concentrated to synapses with a marked reduction at extrasynaptic sites over the exact same developmental time frame. We pinpointed this NRG immunoreactivity to the border zone between the lamina densa and lamina lucida close to the nerve terminal from where it is likely released. This region of the basal lamina has been shown to contain the negatively charged glycosaminoglycan side chains of HSPGs (Kogaya et al., 1990) that may serve to concentrate NRGs. A metabolic labelling study on NMJs formed in culture showed that the HSPG that becomes highly concentrated at synapses was almost exclusively derived from muscle (Swenarchuk et al., 1990). This suggests that the deposition of HSPGs at NMJs may be a selective way by which a given muscle can regulate NRG accumulation at its own synapses.

The exact functional role of this relatively late concentration of NRG at NMJs remains to be determined. Ig-like domain-containing isoforms are clearly important functionally in synapse development and maturation, as mice with specific disruptions of this domain fail to function properly (Sandrock et al., 1997). One possible role of this late appearance comes from an analysis of AChR distribution in embryonic chick posterior latissimus dorsi muscle revealing that a rapid loss of extrajunctional receptors occurs by an activity-dependent process between E17 and E19 (Burden, 1977). This correlates precisely to when both NRG and HSPGs accumulate at endplates with a corresponding reduction in these proteins at extrajunctional sites. The restriction of HSPGs and NRG to

synaptic sites, therefore, may restrict AChR synthesis to muscle subsynaptic nuclei and result in less NRG activity and AChR synthesis at extrajunctional sites. HSPG deposition in the synaptic basal lamina may influence synapse maturation by providing a stable, concentrated source of NRG.

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