Development 124, 3575-3586 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV9542

Isoform-specific expression and function of neuregulin

Dirk Meyer[1,‡,*], Tomoichiro Yamaai[1,*], Alistair Garratt[1,*], Eva Riethmacher-Sonnenberg[1], Darci Kane[2], Lars E. Theill[2] and Carmen Birchmeier[1,†]

[1]Max-Delbrück-Center for Molecular Medicine, Department of Medical Genetics, Robert-Rössle-Str. 10, 13122 Berlin, Germany
[2]Department of Molecular Genetics, Amgen Inc., 1840 Dehavillard Drive, Thousand Oaks, CA 91320-221, USA

[‡]Present address: Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129-2060, USA
*These authors contributed equally to the work
[†]Author for correspondence (e-mail: cbirch@mdc-berlin.de)

SUMMARY

Neuregulin (also known as NDF, heregulin, ARIA, GGF or SMDF), induces cell growth and differentiation. Biological effects of neuregulin are mediated by members of the erbB family of tyrosine kinase receptors. Three major neuregulin isoforms are produced from the gene, which differ substantially in sequence and in overall structure. Here we use in situ hybridization with isoform-specific probes to illustrate the spatially distinct patterns of expression of the isoforms during mouse development. Ablation of the *neuregulin* gene in the mouse has demonstrated multiple and independent functions of this factor in development of both the nervous system and the heart. We show here that targeted mutations that affect different isoforms result in distinct phenotypes, demonstrating that isoforms can take over specific functions in vivo. Type I neuregulin is required for generation of neural crest-derived neurons in cranial ganglia and for trabeculation of the heart ventricle, whereas type III neuregulin plays an important role in the early development of Schwann cells. The complexity of neuregulin functions in development is therefore due to independent roles played by distinct isoforms.

Key words: erbB2, erbB3 and erbB4 receptors, neural crest, Schwann cells, glia, mouse

INTRODUCTION

The *neuregulin* gene encodes various isoforms of a recently identified growth and differentiation factor, which all contain an EGF-like domain. The isoforms differ significantly in their structure and are also known as NDF (neu differentiation factor; Wen et al., 1992), heregulin (Holmes et al., 1992), GGF (glial growth factor; Marchionni et al., 1993), ARIA (acetylcholine receptor inducing activity; Falls et al., 1993) or SMDF (sensory and motor neuron-derived factor; Ho et al., 1995). Various cell types, among them glial, muscle and epithelial cells, can respond to the factor by growth and differentiation in vitro or in vivo (for reviews see Peles and Yarden, 1993; Carraway and Burden, 1995; Marchionni, 1995; Lemke, 1996). Neuregulin-induced cellular responses are mediated by tyrosine kinase receptors of the erbB family: neuregulin binds erbB3 and erbB4 with high affinity, but not the erbB2 (HER2) and erbB1 (EGF) receptors (Peles et al., 1992, 1993; Plowman et al., 1993; Carraway et al., 1994). Binding affinity of neuregulin to erbB3 is increased by the presence of erbB2 (Sliwkowski et al., 1994). Moreover, when co-expressed with erbB3 or erbB4, neuregulin induces tyrosine phosphorylation of the erbB2 receptor (Holmes et al., 1992; Wen et al., 1992; Carraway and Cantley, 1994; Beerli et al., 1995). This is the result of heterodimerization of erbB2 with erbB3 or erbB4 and subsequent receptor cross-phosphorylation.

Experiments in cell culture and mutational analysis in mice demonstrate that neuregulin is important for development of the neural crest (Shah et al., 1994; Meyer and Birchmeier, 1995; Kramer et al., 1996). The neural crest is a transient and migratory cell population that emerges from the dorsal neural tube and gives rise to many different cell types at various locations in the body (LeDouarin, 1982). Neural crest cells from the trunk and head however have distinct development potentials. Neural crest cells in the trunk form the sensory and autonomic nervous system, chromaffin cells of the adrenal medulla and melanocytes. Neural crest cells from the cranial neural tube also contribute to the sensory and autonomic nervous system, but in addition form mesenchymal tissues of the face and skull. Differences also exist in the development of cranial and trunk sensory ganglia. Sensory neurons in cranial ganglia have a mixed origin in that they derive from the neural crest or the ectodermal placode, whereas neurons of dorsal root ganglia are formed by neural crest cells only (Hamburger, 1961, D'Amico, 1983). Schwann cells in the head and trunk are solely formed by the neural crest (LeDouarin, 1982).

Targeted mutations of the murine genes that encode *neuregulin* as well as the *erbB4* and *erbB2* receptors have recently been reported, and an *erbB3* mutation is presently being characterized (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996; D. Riethmacher and C.B., unpublished data). The phenotypes demonstrate multiple essential roles of neuregulin in the formation of the peripheral nervous system and the heart. In embryos lacking sequences important for all neuregulin isoforms, development of cranial ganglia is severely disturbed due to a loss of neural crest-derived neurons, whereas dorsal root ganglia form appropriately. Moreover, precursors of Schwann cells are affected

(Meyer and Birchmeier, 1995). Mutations in *neuregulin* also impair development of the myocardium, which is the likely cause of death of mutant embryos on day 10.5 of development (Meyer and Birchmeier, 1995; Kramer et al., 1996). In the myocardium, similar defects are observed in animals that lack *erbB4*, whereas the development of cranial ganglia is affected in a distinct and subtle manner (Gassmann et al., 1995). Thus, the phenotypes of *erbB4* and *neuregulin* mutants overlap but are not identical, demonstrating that in vivo additional receptor(s) recognize neuregulin. Our analysis of *erbB3* mutant mice indicates that this additional receptor corresponds to erbB3 (D. Riethmacher et al., unpublished data). Moreover, mutation of *erbB2* causes very similar defects as mutation of neuregulin, demonstrating an essential role of erbB2 as a co-receptor for erbB3 and erbB4 (Lee et al., 1995).

An extraordinary variety of different isoforms are produced from the *neuregulin* gene (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Wen et al., 1994; Ho et al., 1995). Common to all isoforms is an EGF-like domain that suffices to elicit biological responses (Holmes et al., 1992). We use the following classification for the neuregulin isoforms (Fig. 1A): (I) type I isoforms (originally identified as NDF/heregulin or ARIA) contain an Ig-like domain, an EGF-like domain (α- and β-variant), a proteolysis site, a hydrophobic domain suggested to act as internal signal sequence for secretion of the factor and additional C-terminal sequences; (II) type II isoforms (originally identified as GGF) contain a signal peptide, a kringle-like sequence plus Ig and EGF-like (β-variant) domains; (III) type III isoforms (originally identified as SMDF) share only the EGF-like domain (β-variant) with other isoforms; notable in the N-terminal part is a hydrophobic domain within a cysteine-rich sequence. The different isoforms of neuregulin are produced from transcripts that arise by alternative splicing and, potentially, by the usage of different promoters. The biological significance of this extraordinary diversity is as yet unexplained. All major isoforms of neuregulin can directly interact with the erbB3 and the erbB4 receptors. It has been demonstrated that type I isoforms show somewhat higher affinity for erbB4 than for erbB3 (Tzahar et al., 1994). In addition, differences in binding affinities of type I neuregulin that contain α- or β-type EGF-like domains are observed (Marikovsky et al., 1995).

The expression pattern of neuregulin has been previously analyzed by in situ hybridization with probes that recognize either all or specific subsets of neuregulin transcripts (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Chen et al., 1994; Meyer and Birchmeier, 1994; Corfas et al., 1995; Ho et al., 1995). We report here an extended and systematic analysis of the expression patterns of the major isoforms (I-III) during mouse development by the use of isoform-specific probes. We demonstrate that type I neuregulin is the predominant isoform expressed in early embryogenesis, whereas type II and type III neuregulins are first detected at midgestation. Distinct and dynamic expression patterns of the isoforms are observed during development of the peripheral and central nervous system, indicating that the isoforms take over distinct functions. Moreover, we show here that mutations in *neuregulin* which affect separate isoforms interfere in a distinct manner with development of the peripheral nervous system. We conclude that type I neuregulin is essential for development of neural crest-derived sensory neurons in cranial ganglia and for trabeculation of the cardiac ventricle, whereas type III is required for development of the Schwann cell lineage. Taking into account the individual function(s) of neuregulin isoforms allows a better understanding of the apparent complex roles of this signaling system in development of the nervous system.

MATERIALS AND METHODS

Mutant mouse strains

The generation of targeted mutations in the mouse *neuregulin* gene (*neuregulin*$^{\Delta EGF\text{-}lacZ}$ and *neuregulin*^{lg} allele) and the establishment of strains that carry the mutations have been described by Meyer and Birchmeier (1995); Kramer et al. (1996). Homozygous mutant animals were obtained by heterozygous matings; the genotype of the embryos and animals was determined by PCR.

In situ hybridization

Frozen sections (10 μm) of mouse embryos at various stages of development were prepared and hybridized with ^{35}S-labeled mRNA probes. Sense and antisense mRNA were synthesized from various DNA clones: (i) neuregulin type I-specific mouse cDNA sequence corresponding to coding segment 2 (nucleotides 170-396 in the reported rat *NDF* cDNA, Wen et al., 1992); (ii) neuregulin type II-specific mouse genomic DNA fragment that encodes coding segment 1 (nucleotides 265-737 in the reported rat *GGF* sequence, Marchionni et al., 1993); this probe also contains an additional 50 bp of intron sequence. To verify the results obtained with this probe, we used a ^{32}P-end labeled synthetic oligonucleotide corresponding to the sense or anti-sense orientation of nucleotides 394-429 in the *GGF* sequence; (iii) neuregulin type III-specific mouse cDNA fragment corresponding to sequences that encode segment *a* (nucleotides 450-1192 in the reported human *SMDF* sequence, Ho et al., 1995); (iv) pan-neuregulin probe corresponding to mouse cDNA encoding coding segments 3-7 and 11 (nucleotides 391-1458 in the reported rat *NDF* cDNA sequence (Wen et al., 1992; see also Meyer and Birchmeier, 1994); (v) mouse *erbB3* cDNAs corresponding to nucleotides 1163 to 2675 in the human sequences (Plowman et al., 1990); this cDNA probe also contained 3′ untranslated sequences; (vi) mouse *erbB4* cDNA encoding the extracellular domain of erbB4; (vii) a 1.8 kb fragment of the rat p75 cDNA (Buck et al., 1988). Labeled transcripts were synthesized either with T3, T7 or Sp6 RNA polymerase and ^{35}S-labeled UTP and CTP at a specific activity of ≥1000 Ci/mmol (Amersham). Hybridization conditions were essentially as described by Sonnenberg et al. (1991) except that hybridization with the type II-specific probe was performed in 55% instead of 50% formamide. Slides were dipped in Kodak NTB-2 emulsion; sections hybridized with riboprobes were exposed for 2 weeks at 4°C. All probes revealed reproducible hybridization patterns on sections when used in antisense orientation. Transcripts in sense orientation revealed no specific hybridization patterns. For whole-mount hybridizations, digoxigenin-labeled mRNA probes were synthesized by the use of a digoxigenin labeling kit (Boehringer Mannheim). Digoxigenin-labeled type III and pan-neuregulin probes generated signals, but not the short type I probe. This was attributed to a low sensitivity of this probe. Stained embryos were cleared in 75% glycerol, 25% PBS and photographed under a Zeiss microscope.

X-gal staining, histological and immunohistochemical analysis

E6.5 to E11 embryos heterozygous for *neuregulin*$^{\Delta EGF\text{-}lacZ}$ were dissected, fixed for 15 minutes in 1 part 4% (w/v) paraformaldehyde (PFA) and 3 parts solution A (0.1 M $KHPO_4$, pH 7.4, 2 mM $MgCl_2$, 5 mM EGTA). E12 and E18 embryos were dissected, embedded in OCT compound (Tissue-Tek, Miles Inc. Elkhart, Illinois, USA) at –7°C. 10-20 μm sections were cut, and fixed for 5 minutes as

described above. Embryos or cryosections were washed twice for 5 minutes in solution A containing 0.02% (w/v) NP-40, and expression of β-galactosidase was visualized by incubation at 37°C in solution A containing 0.02% (w/v) NP-40, 5 mM hexacyanoferrate(III), 5 mM hexacyanoferrate(II), and 0.5 mg/ml X-gal. X-gal precipitate was viewed on whole-mount-stained embryos (E6.5-11-5), or after sectioning of stained embryos embedded in Technovit 7100 resin (Kulzer GmbH, Wehrheim, Germany). For counterstaining, eosin/haematoxilin or borax carmine were used.

For immunohistochemistry, fixed E10.5 embryos were bleached for 1 hour on ice in 3:1 methanol/hydrogen peroxide (30% solution in water), rehydrated, washed in PBS containing 0.1% Tween-20 (PBT), treated with Proteinase K (20 μg/ml) for 5 minutes and refixed. Embryos were then incubated overnight at 4°C in 1% serum and mouse monoclonal anti-NF160 antibody (1:1000; Sigma), washed for 1 day in PBT, incubated overnight at 4°C in PBT containing 1% serum and rabbit anti-mouse IgG peroxidase conjugate (1:1000, Sigma). The color reaction was developed by incubation in 0.3 mg/ml diaminobenzidine, 0.03% H_2O_2, 0.05% $NiCl_2$ and stopped by washing in PBT.

RESULTS

Isoform-specific expression of *neuregulin* during early mouse development

Three major isoforms with distinct overall sequence and structure are produced by the *neuregulin* gene (Fig. 1A). Isoform-specific sequences were used to determine the distribution of the corresponding mRNAs by in situ hybridization: (i) sequences of coding segment 2 for type I isoforms (coding segments and exons present in type I transcripts are indicated in yellow in Fig. 1B); (ii) exon 1 for type II isoform (indicated in green); (iii) coding segment *a* for type III isoforms (indicated in blue, cf. also Materials and Methods). A long fragment which contains sequences present in all isoforms was used as a pan-neuregulin probe. Antisense ^{35}S-labeled transcripts of all four probes generated reproducible hybridization signals on sections; the corresponding sense transcripts generated no signal above background. Digoxigenin-labeled type II, type III and pan-neuregulin transcripts were also used for whole-mount in situ hybridizations; type I transcripts generated no signals in whole-mount hybridizations, which was attributed to a low sensitivity of this very short probe.

An independent method for examining neuregulin type I expression was provided by our previously described *neuregulin-lacZ* fusion allele in mice, *neuregulin*$^{\Delta EGF\text{-}lacZ}$ (Meyer and Birchmeier, 1995). In this allele, *lacZ* was introduced by homologous recombination in frame into coding segment 6 present in all isoforms (Fig. 1B). Active β-galactosidase was visualized by staining embryos heterozygous for *neuregulin*$^{\Delta EGF\text{-}lacZ}$ with X-gal, and surprisingly was detected only at sites of type I neuregulin expression. This is due to the fact that type II and type III neuregulin-β-galactosidase fusion proteins contain hydrophobic sequences that serve as signal sequences (see Fig. 1A) and are directed to the secretory pathway where β-galactosidase is inactive (cf. Skarnes et al., 1995). In contrast, type I neuregulin-β-galactosidase fusion proteins do not contain such hydrophobic signal sequences and therefore remain in the cytoplasm and enzymatically active.

With these tools, we found that during early postimplantation development up to day 10, only type I neuregulin isoforms are expressed, and identical patterns of expression were observed by analysis of β-gal activity and in situ hybridization (Fig. 2). On E6.5 a broad expression was observed, which on E7.5 was restricted to the rostral portion of the embryo (Fig. 2A,B). Sections demonstrated that staining was associated with undifferentiated cells of mesenchymal appearance, i.e. the mesoderm. On E8, expression was found in the hindbrain (rhombomeres 2, 4) and the eye (Fig. 2C,D). The signal in the eye was located at the eye stalk and optic placode on sections (not shown). In the head, an apparent remnant of the earlier mesodermal signal is also observed. On E8.5 additional sites of expression are rhombomere 6, the dorsal part of the spinal cord, cartilage in the branchial arches and the heart (Fig. 2E,F); in sections, the signal in the heart originated in the endocardium of the ventricle (compare also Fig. 3I). We conclude that cells derived from several lineages (mesoderm, neuroectoderm, placode and neural crest) express neuregulin type I during early postimplantation development.

Isoform-specific expression of neuregulins during development of sensory ganglia and the spinal cord

In later stages, we determined in detail the expression of neuregulin isoforms in developing cranial ganglia. Neuregulin type I is expressed in the trigeminal ganglion on E9.5 or E10 (Fig. 3A,D, indicated by t); in sections, this ganglion is well delineated and the large stained cells have the round shape characteristic of developing neurons (Fig. 3G). Signals are also seen at or close to the sites where the geniculate ganglion, glossopharyngeal and vagal ganglia form on E9.5 (arrowheads in Fig. 3A); these ganglia are however not well delineated at this stage (see Fig. 3H) and the stained cells have an undifferentiated mesenchymal appearance. Remarkably, an additional isoform, neuregulin type III, appears the first time on E10, and is expressed in cranial ganglia, dorsal root ganglia and in developing motoneurons located in the ventral column of the spinal cord (Fig. 3F, compare with C). Pan-neuregulin probes reveal signals due to type I and type III isoforms (Fig. 3E, compare with D,F, compare also Fig. 4J), although the signals generated by type III transcripts dominate the pattern. We conclude that during formation of cranial ganglia type I and type III neuregulins are expressed; note that type I precedes type III. Expression of the neuregulin receptors erbB3 and erbB4 during these stages has previously been reported (Meyer and Birchmeier, 1995; Gassmann et al., 1995); erbB3 but not erbB4 is expressed in the neural crest cells and in developing cranial ganglia.

We analyzed the expression of neuregulin isoforms in more detail in the developing spinal cord and dorsal root ganglia (Fig. 4). On E10, type I neuregulin expression was detected by β-gal activity or in situ hybridization in a well defined stripe in the dorsal spinal cord (arrowheads in Fig. 4A,D). This is a transient signal not found on E12 (Fig. 4B,E). A new site of type I expression is observed at later stages (e.g. E18): a few neurons that are scattered in dorsal root ganglia (arrowheads in Fig. 4C,F). The first type II-specific signal during development is detected on E10 in the notochord (see arrowhead in Fig. 4G). On E12 and E18, type II transcripts are also observed in the nervous system, i.e. in the spinal cord and in dorsal root ganglia (Fig. 4H,I). Low levels of expression are also found in the skeletal muscles (indicated by m). Strong type III neuregulin expression is observed during all of these stages (E10-E18) in

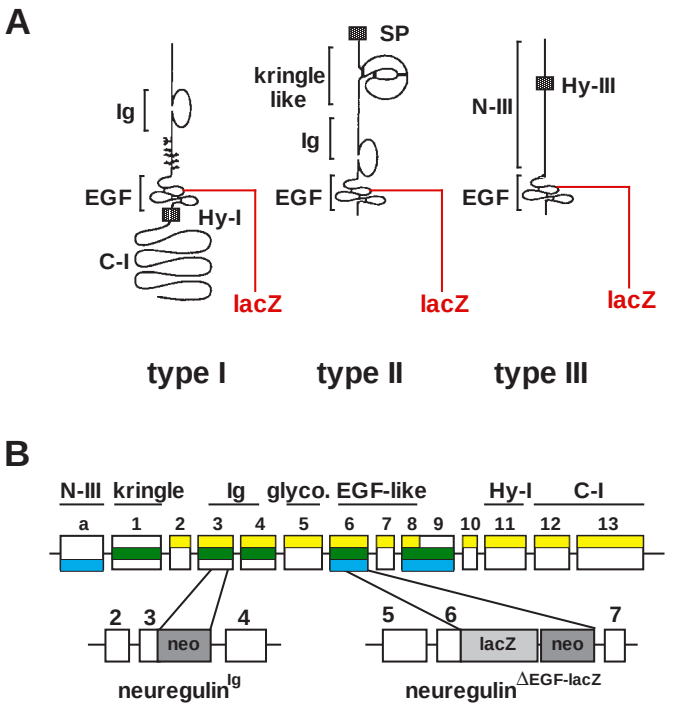

Fig. 1. Schematic structure of the three major protein isoforms of neuregulin and of the neuregulin gene. (A) Structure of neuregulins type I (neu differentiation factor, NDF/heregulin, HRG), type II (glial growth factor, GGF), and type III (sensory and motor neuron-derived factor, SMDF). Characteristic domains present in the proteins are indicated. *EGF*: EGF-like domain present in all isoforms; *Ig*: Ig-like domain present in type I and type II neuregulins; *SP*: signal peptide present in type II neuregulin; *kringle-like* domain present in type II neuregulin; *Hy I and Hy III*: internal hydrophobic sequence stretches of type I and type III neuregulins, respectively; *N-III*: unique N-terminal domain of type III neuregulin; *C-I*; unique C-terminal domain of type I. The point of fusion between the neuregulin and β-galactosidase in the different isoforms produced from the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ allele is indicated. (B) Hypothetical structure of the *neuregulin* gene (cf Marchionni et al., 1993). The figure represents a composite of the knowledge of the mammalian neuregulin gene based on the partial analysis of the mouse and bovine genomic DNA and of various cDNA sequences. Coding segments 1, 3, 4 and 6-11 have been characterized as exons in bovine or mouse DNA (Marchionni et al., 1993; Meyer and Birchmeier, 1995; Kramer et al., 1995); all other segments were deduced from cDNA sequence comparisons. Genomic mapping has to resolve the order of coding segments a, 1 and 2; the relative order of all other segments can be inferred from cDNA sequences. Domains encoded by different segments of the gene are indicated. Segments encoding the isoforms are marked in yellow (type I), green (type II) and blue (type III). Type I neuregulin (yellow) is encoded by segments 2-6, 7 or 8, 10-13; 7 or 8 encode the alternative C-terminal part of the EGF-like domain found in the α- or β-type of this isoform; segments 5, 10 or 12 are optional and are not always present. Type II (green) is encoded by segments 1, 3, 4,6, 8, 9; segment 5 is optional and sometimes included. Type III (blue) is encoded by segments a, 6, 8 and 9. The genomic structure of two mutant alleles, *neuregulin*^{Ig} and *neuregulin*$^{\Delta EGF\text{-}lacZ}$, are depicted. *Neuregulin*^{Ig} contains an insertion of a *neo*^{res} gene cassette in exon 3, which causes premature termination of type I and type II neuregulins, but leaves all coding segments of type III intact. *LacZ* and *neo*^{res} sequences are inserted in exon 6 of the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ allele which is present in all isoforms. Therefore all types of neuregulin are affected by this mutation.

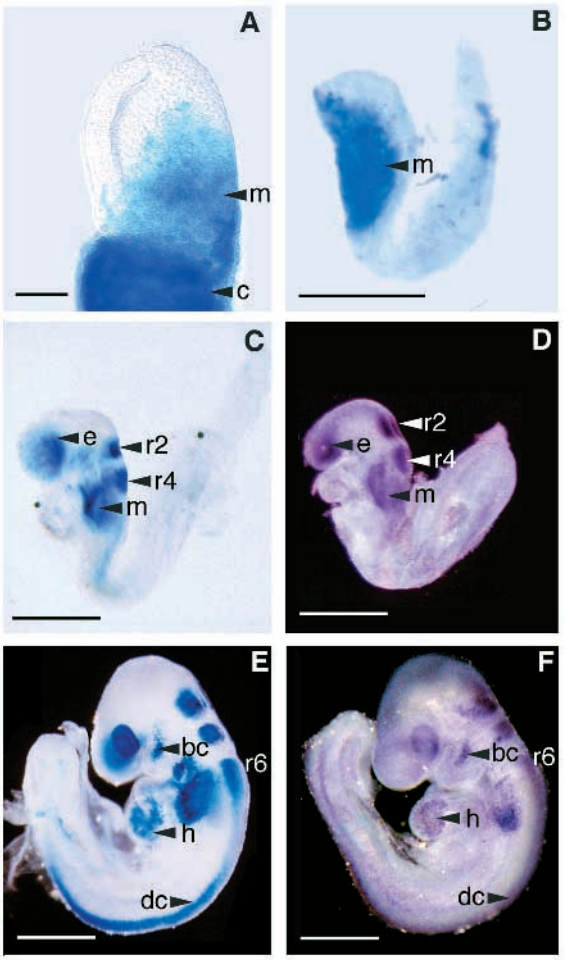

Fig. 2. Expression of neuregulin during early mouse development. Expression of neuregulin during early mouse development as determined by staining for β-galactosidase activity in animals heterozygous for the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ allele (A-C,E) and by whole-mount hybridization with a pan-neuregulin probe (D,F). The age of the embryos is E6.5 (A), E7.5 (B), E8 (C,D) and E8.5 (E,F). Note that the patterns determined by β-galactosidase activity and by hybridization with a pan-probe are identical in E8 (C,D) and E8.5 (E,F) embryos. bc, branchial arch cartilage; c, ectoplacental cone; dc, dorsal neural tube; e, eye; h, heart; m, mesoderm; r2,r4, r6, rhombomeres 2, 4 and 6. Bar in A corresponds to 100 μm, bars in B-F to 500 μm.

motoneurons located in the ventral column of the spinal cord and in dorsal root ganglia (Fig. 4J-L, arrowheads point to dorsal root ganglia and motoneurons). At later stages, a broader distribution of type III transcripts is detected. Thus we observe a dynamic and distinct pattern of expression of all three neuregulin isoforms during development of the spinal cord and dorsal root ganglia.

We next analyzed the pattern of expression of erbB3 and erbB4 to correlate this with the expression of neuregulin isoforms. Interestingly, erbB4 and erbB3 are expressed predominantly in the central and peripheral nervous system, respectively (Fig. 4M-R). Transcripts for erbB4 are found in two stripes in the spinal cord on E10 (indicated by arrows in Fig. 4M). A strong signal is observed in the ventral portion on E12 (Fig. 4N), and in the entire spinal cord on E18 (Fig. 4O).

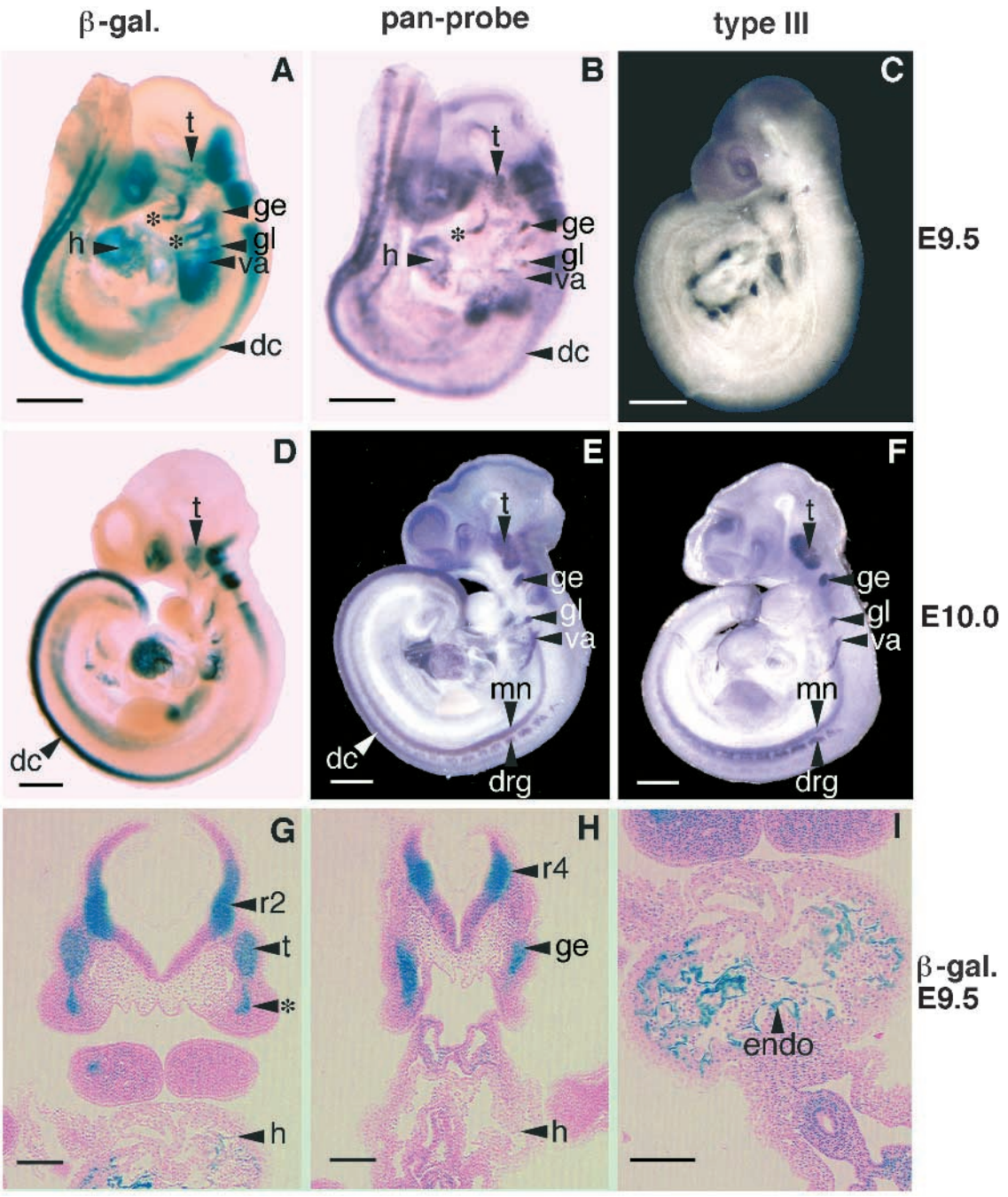

Fig. 3. Expression of neuregulin isoforms during formation of sensory ganglia. Embryos on E9.5 (A-C) and E10 (D-F) were analyzed for neuregulin expression. Embryos heterozygous for *neuregulin*$^{\Delta EGF\text{-}lacZ}$ were analyzed by staining for β-galactosidase activity (A,D). Wild-type animals were analyzed by hybridization with a pan-neuregulin (B,E) or a type III-specific probe (C,F). Sections of an E9.5 embryo heterozygous for *neuregulin*$^{\Delta EGF\text{-}lacZ}$ analyzed for β-galactosidase activity (G-I). Cranial ganglia (t, trigeminal; ge, geniculate; gl, glossopharyngeal; va, vagal ganglia); dc, dorsal spinal cord; drg, dorsal root ganglia; endo, endocardium; h, heart; motoneurons located in the ventral column of the spinal cord (mn); the asterisks point towards branchial arch cartilage. Bars in A-F correspond to 500 μm, bars in G-I to 200 μm.

Weak expression of erbB4 is found also in skeletal muscles. Strong erbB3 expression is associated with dorsal root ganglia, with cells that line all developing axons in the periphery and with skeletal muscles (Fig. 4 P-R). Because of their location, we assign erbB3-expressing cells associated with axons as developing Schwann cells (see also below).

Neuregulin isoforms have distinct functions in development: appropriate formation of Schwann cell precursors requires type III neuregulin

The differential expression of neuregulin isoforms during development of the peripheral nervous system enticed us to compare phenotypes resulting from previously generated

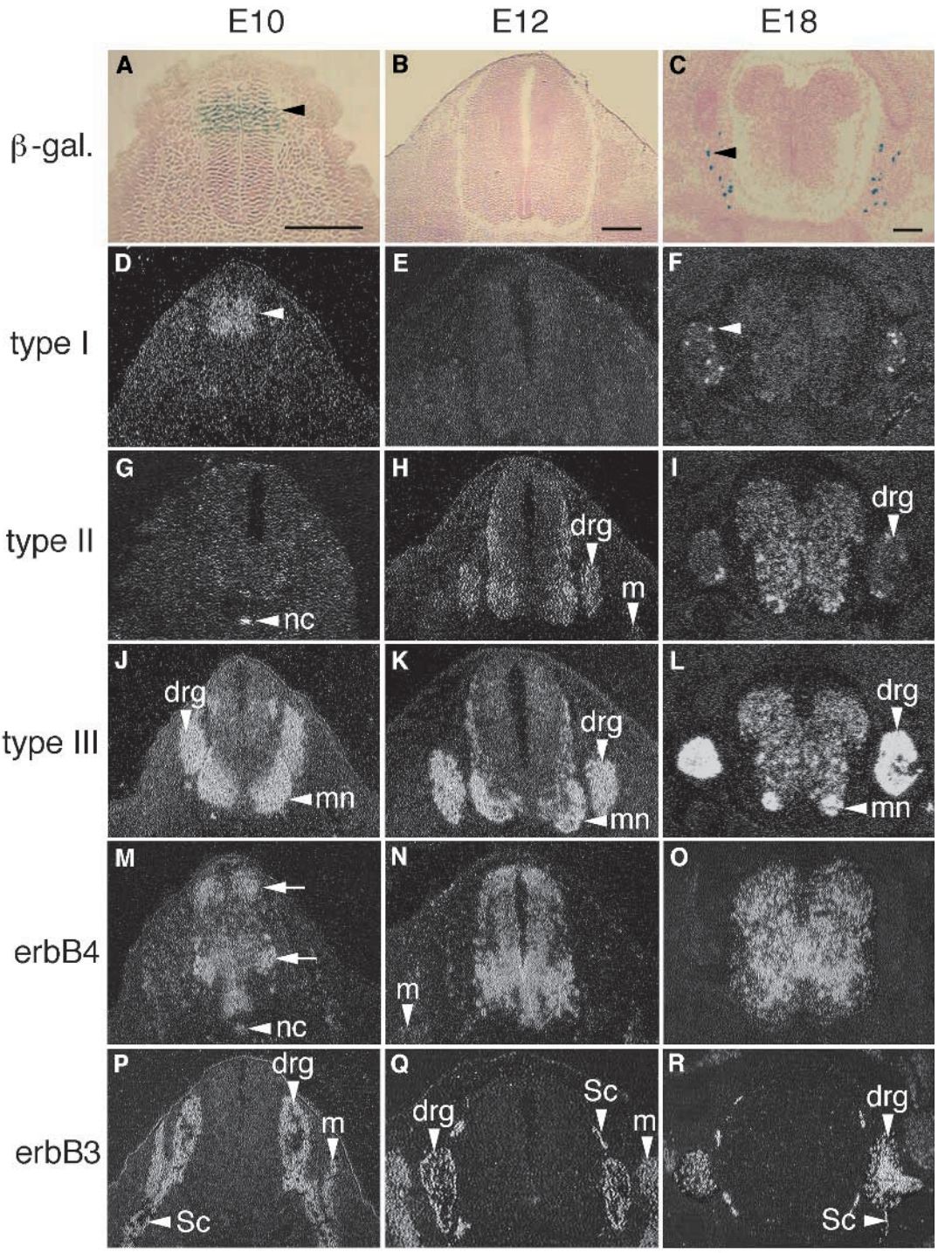

Fig. 4. Expression of neuregulin isoforms and their receptors, erbB3 and erbB4, during spinal cord and dorsal root ganglia development. Mouse embryos at three stages of development, E10 (A,D,G,J,M,P), E12 (B,E,H,K,N,Q), E18 (C,F,I,L,O,R) were sectioned and analyzed for neuregulin, erbB3 or erbB4 expression. Expression of type I neuregulin as revealed by staining for β-galactosidase activity in animals heterozygous for the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ (A-C) or by hybridization with a type I-specific probe (D-F). Note that both patterns are identical. Arrowheads in A and D indicate a signal in the dorsal column of the spinal cord, and in C and F a signal associated with scattered neurons in dorsal root ganglia. (G-I) Expression of type II and (J-L) type III neuregulins as revealed by hybridization with isoform-specific probes. Expression of the erbB4 (M-O) and the erbB3 (P-R) receptors were determined by in situ hybridizations. Arrows in M indicate two stripes of erbB4-positive cells in the spinal cord. Also, indicated are dorsal root ganglia (drg), notochord (nc), myotome or skeletal muscle (m), motoneurons located in the ventral column of the spinal cord (mn) and Schwann cells (Sc) and their precursors that line the the spinal nerves and dorsal roots. Bar in A corresponds to 500 μm, and bars in B,C to 200 μm.

mutant neuregulin alleles. Two alleles were compared (Fig. 1B): (i) The *neuregulin*$^{\Delta EGF\text{-}lacZ}$ allele lacks sequences encoding the EGF-like domain which are essential for the function of all neuregulin isoforms; (ii) the *neuregulin*^{Ig} allele contains an insertion within an exon encoding the Ig-like domain present only in type I and II neuregulin, but type III coding segments are not altered. The two alleles, *neuregulin*$^{\Delta EGF\text{-}lacZ}$ and *neuregulin*^{Ig}, therefore produce, respectively, no functional neuregulin isoforms or type III neuregulin only. No phenotypic difference between the two mutations has previously been described. Both mutant alleles cause embryonal death on E10.5 due to defects in heart development, specifically a defect of trabeculation of the ventricle.

Moreover, a similar loss of neurons in the cranial ganglia is observed in *neuregulin*$^{\Delta EGF\text{-}lacZ}$ and *neuregulin*^{Ig} embryos (Fig. 5A-C; cf. Meyer and Birchmeier, 1995; Kramer et al., 1996). However, we report here a major difference in the phenotypes of the two mutations which assigns a distinct role for type III neuregulin. By using *erbB3* or *p75* (encoding the low molecular weight NGF receptor) cDNA as hybridization probes, we visualized Schwann cell precursors (Figs 5D-I, 6). In situ hybridization with *erbB3* of E10 embryos revealed

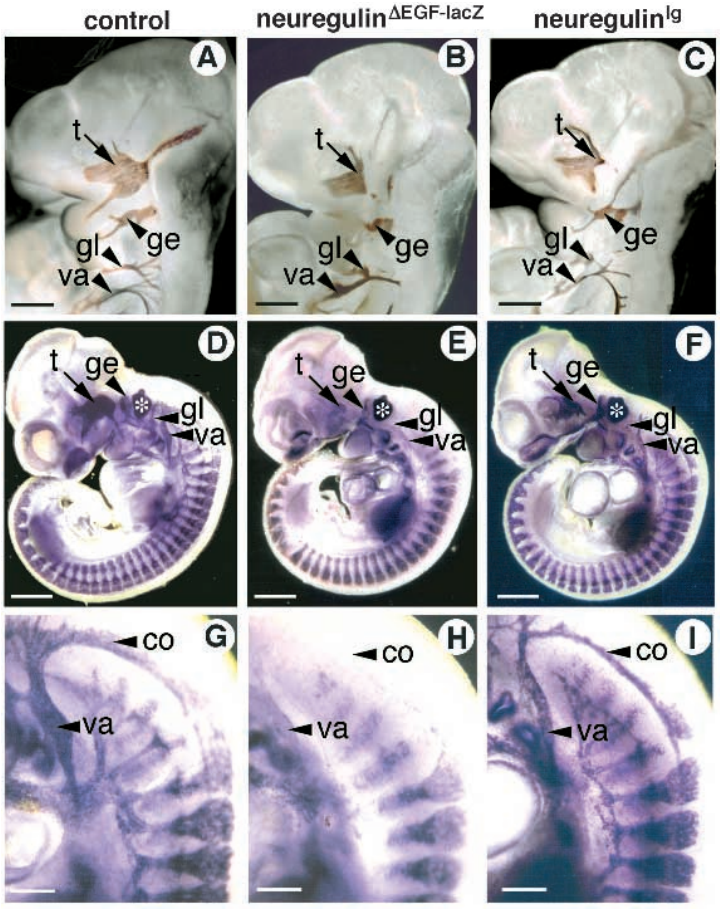

Fig. 5. Isoform-specific function of type type I and III neuregulins during cranial ganglia development. Appearance of cranial ganglia and cranial nerves of control (A) and homozygous mutant embryos that carry the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ (B) or *neuregulin*^{Ig} allele (C), as visualized by anti-neurofilament (NF160) antibody on E10.5 of development. Whole-mount hybridization with erbB3-specific probes in control embryos (D,G), homozygous mutant embryos that carry the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ allele (E,H), and homozygous mutant embryos that carry the *neuregulin*^{Ig} allele (F,I). Depicted are entire embryos (D-F) and areas of the neck (G-I). Note the *erbB3*-expressing cells that associate with peripheral nerves, i.e. Schwann cell precursors. Indicated are cranial ganglia (trigeminal (t), geniculate (ge), glossopharyngeal (gl), vagal (va), commissural (co) ganglia); the otic vesicle is indicated by an asterisk. Bars: 800 μm in A-C, 500 μm in D-F; 200 μm in G-I.

Schwann cell precursors that line cranial nerves in control or homozygous *neuregulin*^{Ig} mutant embryos; these cells are absent in homozygous *neuregulin*$^{\Delta EGF\text{-}lacZ}$ embryos (compare Fig. 5D,F with E and G,I with H). Sections demonstrate that *erbB3* or *p75* expressing Schwann cell precursors that line spinal nerves in the trunk also appear normal in *neuregulin*^{Ig} embryos whereas their numbers are reduced in *neuregu-lin*$^{\Delta EGF\text{-}lacZ}$ embryos (compare Fig. 6B with A,C or Fig. 6E with D,F). These differences in the phenotypes demonstrate that type III neuregulin is required for development of Schwann cell precursors.

Expression of neuregulin isoforms in the developing brain

Complex and isoform-specific expression patterns of neuregulin were also observed during brain development (Fig. 7). On E18, type I neuregulin is expressed in the ventricular zone of the telencephalon and the superior or inferior colliculus (Fig. 7A,B). At earlier stages, expression in the ventricular zone can also be observed (Table 1). Type II transcripts are found at low levels in all parts of the brain; high signal intensities are associated with particular structures such as the ventricular zone and claustrum of the telencephalon and the retina (Fig. 7C). In earlier development (E12), neuregulin type II transcripts are found in the subventricular zone of the neuroepithelium (Table 1). Type III neuregulin is expressed in the entire brain; particularly strong signals are observed at several sites, for instance the hippocampus, nuclei of the thalamus and the mesencephalon, retina and pia mater (Fig. 7D). Expression is also detected in the olfactory epithelium. On E12, type III neuregulin is expressed in the outermost layer of the telencephalon (Table 1). Thus, type I neuregulin is restricted to few cells, in contrast to type II and type III neuregulins, which are expressed broadly.

In the brain, the erbB4, but not the erbB3 receptor is strongly and broadly expressed (Fig. 7E,F; see also Lai and Lemke, 1991). Major signals are for instance associated with the cortical plate, ventricular zone and claustrum in the telencephalon, as well as various nuclei of the mesencephalon (Fig. 7E). During early development, erbB4 transcripts are first detected in rhombomeres 3 and 5 (see also Gassmann et al., 1995; Meyer and Birchmeier, 1995). In contrast, erbB3 transcripts were only detected at two sites in the brain, nuclei of the thalamus (asterisk in Fig. 7F) and

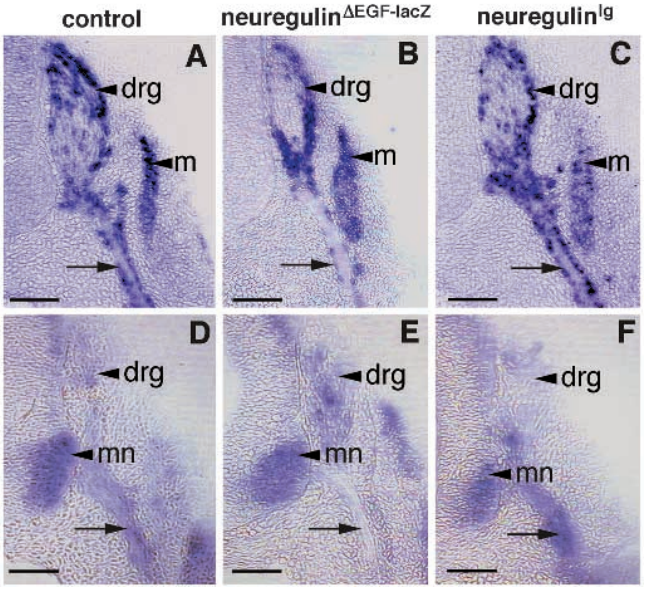

Fig. 6. Isoform specific function of type III neuregulin during Schwann cell development. Schwann cell precursors that line the spinal nerves of control embryos (A,D) and homozygous mutant embryos that carry the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ (B,E) or *neuregulin*^{Ig} allele (C,F) were visualized on E10.5 of development by in situ hybridization with *erbB3* (A-C) or *p75* (D-F) specific probes. Arrows indicate the Schwann cell precursors that line the spinal nerve. drg, dorsal root ganglion; m, myotome; mn, motoneurons located in the ventral horn of the spinal cord. Bars correspond to 50 μm.

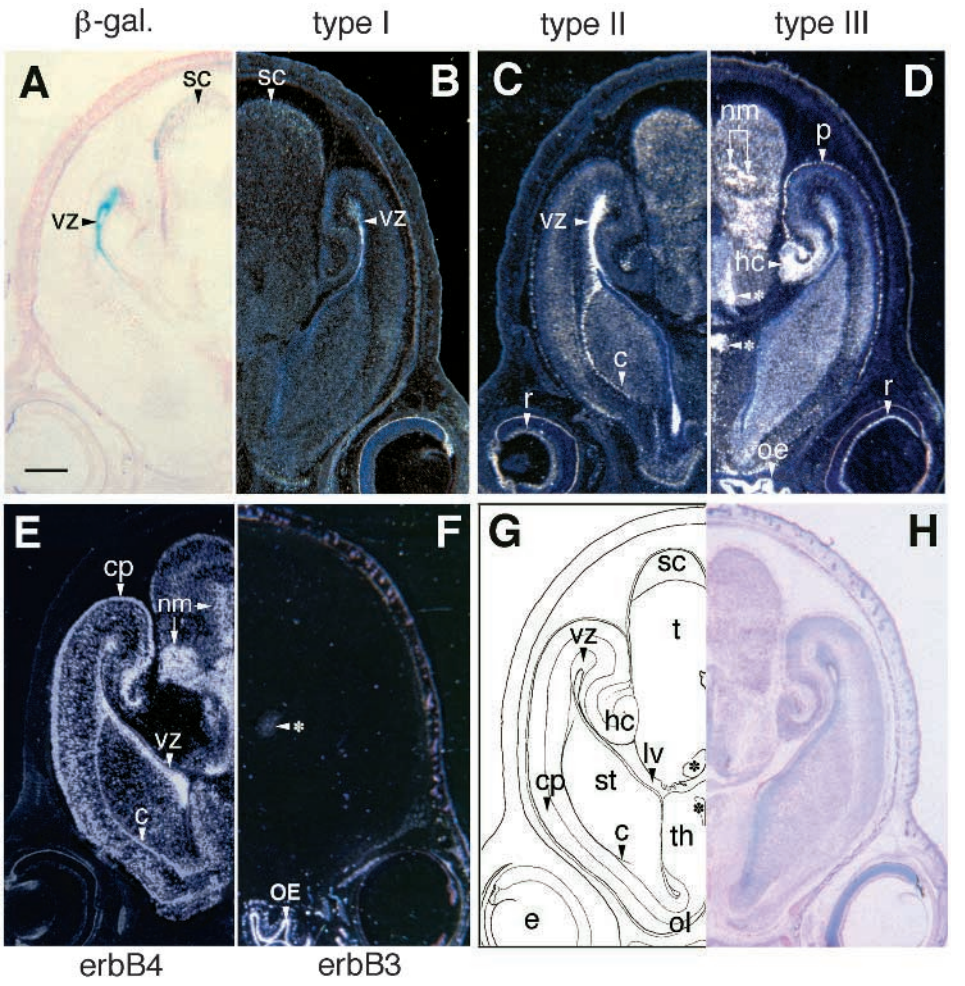

Fig. 7. Expression of neuregulin isoforms and their receptors, erbB3 and erbB4, in the perinatal brain. Cranial transverse sections of mouse embryos on E18 were analyzed for neuregulin, erbB3 or erbB4 expression. Expression of type I neuregulin as revealed by staining for β-galactosidase activity in animals heterozygous for the *neuregulin*$^{\Delta EGF\text{-}lacZ}$ (A) or in wild-type animals by hybridization with a neuregulin type I-specific probe (B). Note that the patterns are identical. Expression of type II (C) and type III (D) neuregulins as revealed by hybridization with isoform-specific probes. Expression of the erbB4 (E) and the erbB3 (F) receptors were determined by in situ hybridizations. (G) A schematic drawing showing different brain structures in the sections and (H) a section stained with haematoxylin/eosin. c, claustrum; cp, cortical plate; e, eye; hc, hippocampus; lv, lateral ventricle; nm, nuclei of the mesencephalon; oe, olfactory epithelium; ol, olfactory lobe ; p, pia mater; r, retina; sc, superior colliculus; st, striatum; t, tectum; th, thalamus; vz, ventricular zone. Asterisks indicate nuclei of the thalamus. Note that the melanin containing retinal pigment layer of the eye can be visualized in dark-field although silver grains are not associated with this cell type. The bar in A corresponds to 1 mm.

the olfactory lobe (not shown). The olfactory epithelium strongly expresses the receptor. During earlier stages, erbB3 is not expressed in the developing brain (not shown).

Therefore we observe very complex expression patterns of neuregulin isoforms and neuregulin receptors during development of the nervous system, skeletal muscles and the heart; further details about the expression of all isoforms and the erbB3 and erbB4 receptors during various developmental stages are given in Table 1.

DISCUSSION

Our phenotype analyses of mutations in mice that ablate different neuregulin isoforms, together with a systematic examination of isoform-specific expression patterns, allow us to assign specific functions to type I and type III neuregulin in mouse development. Type I is the first neuregulin isoform produced in development. Type I neuregulin is expressed in cephalic mesenchyme and cranial ganglia as well as the endocardium and governs development of neural crest-derived neurons and trabeculation of the cardiac ventricle (cf also Meyer and Birchmeier, 1995; Kramer et al., 1996). In contrast, type III neuregulin is expressed in differentiating sensory and motor neurons and acts on the Schwann cell precursors, driving their initial development. This was directly demonstrated by the use of a *neuregulin* allele which produces type III, but not type I or type II neuregulin (Kramer et al., 1996). Genetic analysis has not as yet revealed essential roles of type II neuregulin, which is expressed broadly in the nervous system in the perinatal period.

Isoform-specific expression of neuregulin

We demonstrate here that all three major neuregulin isoforms are expressed in extremely dynamic patterns during development. Important expression sites are: (i) the endocardium of the developing ventricle, which expresses type I (and low levels of type III) neuregulin that acts on the myocardium to induce trabeculation; (ii) cranial ganglia and undifferentiated cells at sites of cranial ganglia formation, which express type I neuregulin that acts on neural crest-derived cells and allows survival and/or development of crest-derived neurons in cranial ganglia; (iii) motoneurons and cells in cranial and dorsal root ganglia, which express type III neuregulin that acts on Schwann cell precursors. Many of the sites which we have identified as expressing particular isoforms have been described previously (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Chen et al., 1994; Meyer and Birchmeier, 1994; Corfas et al., 1995; Ho et al., 1995). However, the hybridization probes used in the majority of these studies contained sequences present in all isoforms and therefore the observed patterns reflect the sum of expression of all neuregulin isoforms. Exceptions are analyses performed with sequences

Table 1. Expression of neuregulin isoforms, the erbB3 and erbB4 receptors in development of the nervous system, skeletal and heart muscle

	Neuregulin type I			Neuregulin type II			Neuregulin type III			erbB3 receptor			erbB4 receptor		
	E12	E15	E18	E12	E15	E18	E12	E15	E18	E12	E15	E18	E12	E15	E18
Telencephalon															
ventricular zone	+	+	+	++	++	++	–	++	++	–	–	–	–	–	++
cortex	–	–	–	+	+	+	+	++	++	–	–	–	++	++	++
hippocampus	o	o	–	o	o	–	o	o	++	o	o	–	o	o	++
olfactory lobes	ND	ND	+	+	+	+	ND	+	+	–	–	+	++	++	++
striatum	o	–	–	o	+	++	o	+	++	o	–	–	o	++	+
claustrum	o	o	–	o	o	++	o	o	–	o	o	–	o	o	++
Di- and mesencephalon	–	+[1]	+[1]	+	+	++[1]	+	++[1]	+[1]	–	–	+[1]	++	++	++
Cerebellum															
cortex	o	o	–	o	o	–	o	o	–	o	o	–	o	o	++
medulla	o	o	–	o	o	+	o	o	++	o	o	–	o	o	++
Pons and medulla oblongata	–	–	–	+	+	+	+[1]	++[1]	++[1]	–	–	–	++	++	++
Spinal cord	–	+	+	+	++	++	++	++	++	–	–	–	++	++	++
Choroid plexus	o	–	–	o	–	–	o	–	–	o	–	–	o	–	–
Pituitary	o	–	–	o	++	++	o	++	++	o	+	–	o	+	++
Nasal cavity															
undifferentiated epithelium	–	o	o	–	o	o	++	o	o	++	o	o	–	o	o
olfactory epithelium	o	–	–	o	–	–	o	++	++	o	++	++	o	–	–
respiratory epithelium	o	–	+	o	–	–	o	–	–	o	++	++	o	–	–
Retina															
undifferentiated epithelium	–	o	o	–	o	o	–	o	o	–	o	o	–	o	o
nuclear layers	o	–	–	o	–	–	o	–	–	o	–	–	o	–	–
ganglion cell layer	o	–	–	o	–	+	o	+	+	o	–	–	o	–	+
Inner ear															
undifferentiated epithelium	–	o	o	–	o	o	–	o	o	–	o	o	+	o	o
sensory epithelium	o	–	–	o	–	–	o	–	–	o	+	+	o	–	+
non–sensory epithelium	o	–	–	o	–	–	o	–	–	o	+	+	o	–	–
Sympathetic ganglia	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–
Skeletal muscle	–	–	–	+	+	+	–	–	–	++	++	+	+	+	+/–
Heart															
myocardium	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++
cushion or valves	–	–	–	–	–	–	–	–	–	–	++	+	+	–	+	++
endocardium	+[2]	+[2]	+[2]	–	–	–	+[3]	+[3]	+[3]	–	–	–	–	–	–

In situ hybridizations were performed on sections of E12, E15 and E18 embryos as described in Material and Methods. All sections were exposed for 2 weeks. Relative signal intensities of each probe associated with different anatomical structures are compared: – no signal; + low signal intensity; ++ high signal intensity. o Not applicable, immature structure; ND not determined; [1]nuclei within the brain structure express neuregulin; [2]endothelia of the yolk sac and other sites of the embryo also express this isoform; [3]signal intensity observed with type III neuregulin probe is low in the endocardium, and was not observed by whole mount in situ hybridizations.

encoding the Ig-domain, which detect the sum of expression of type I and II neuregulins and a study performed with sequences specific for type III (SMDF), which reports similar data to that presented here (Chen et al., 1994; Corfas et al., 1995; Ho et al., 1995). The distinct expression patterns of the three neuregulin isoforms support the hypothesis that different promoters are used for the different transcripts.

A *neuregulin-lacZ* fusion gene (*neuregulin$^{\Delta EGF\text{-}lacZ}$*) that replaces coding sequences for the EGF domain by *lacZ* (Meyer and Birchmeier, 1995) was also useful in our expression analysis. Comparison of patterns of the isoform-specific hybridization signals and β-gal activity demonstrates isoform-specific staining: β-gal activity was not detected at sites of expression of type II or type III neuregulins, for instance motoneurons, but was found at all sites where we detect neuregulin type I expression by in situ hybridization. This is due to the fact that only type I *neuregulin-lacZ* transcripts give rise to active β-galactosidase. Type II and type III β-galactosidase fusion proteins are directed to the secretory compartment; such fusion proteins have been shown to be enzymatically inactive and remain inside the endoplasmic reticulum (Skarnes et al., 1995).

Functional roles of neuregulin isoforms

The assignment of specific functions to type I and type III neuregulin isoforms was possible by comparison of phenotypes of distinct targeted mutations, *neuregulin$^{\Delta EGF\text{-}lacZ}$* and *neuregulin^{Ig}* (Meyer and Birchmeier, 1995; Kramer et al., 1996). The *neuregulin$^{\Delta EGF\text{-}lacZ}$* allele lacks coding sequences essential for all known isoforms, i.e. the EGF-like domain; therefore all neuregulin isoforms are non-functional in animals that carry this mutation. Three phenotypes are observed in these animals: impairment of Schwann cell development, loss of neural crest-derived neurons in cranial ganglia and lack of trabeculation in the heart ventricle (Meyer and Birchmeier, 1995). In contrast, the *neuregulin^{Ig}* mutation causes premature translational termination of isoforms that contain the Ig domain, i.e. type I and type II neuregulins, but does not affect type III coding sequences (Kramer et al., 1996). Loss of neurons in cranial sensory ganglia and absence of trabeculation in the heart was previously observed in embryos that carry the homozygous *neuregulin^{Ig}* allele, but development of Schwann cells had not been analyzed (Kramer et al., 1996). We demonstrate here that Schwann cells develop appropriately in such animals, and

conclude that type III neuregulin suffices for an appropriate development of Schwann cell precursors. Two isoforms, type I or type II, are affected by the *neuregulin^{Ig}* and the *neuregulin$^{\Delta EGF\text{-}lacZ}$* mutations and could theoretically perform functions in the development of cranial ganglia and the heart. Since only one of these two isoforms, type I, is produced during relevant times in the heart and at sites of cranial ganglia formation, we assign these functions to type I neuregulin.

The complex expression patterns of neuregulin isoforms during development suggests that these variants take over many functions in vivo. We show here that neuregulin type III is essential for the appropriate development of Schwann cell precursors that line peripheral nerves. We identify these cells by the following criteria. They express *erbB3* and *p75*, i.e. two genes expressed in the neural crest and its derivatives (Lemke and Chao, 1988; Shah et al., 1994; Meyer and Birchmeier, 1995; Wang and Anderson, 1997), and they associate with peripheral nerves on E10 and at later stages of development (cf. Fig. 4, P-R). Neuregulin is produced by motor and sensory neurons during their entire development, which might allow independent functions at various stages or a continuous trophic role in Schwann cell development. Experiments in cell culture demonstrate that neuregulin affects Schwann cell precursors, immature and mature Schwann cells, and influences cell fate decisions, growth, survival and maturation of cells in this lineage (Marchionni et al., 1993; Shah et al., 1994; Dong et al., 1995; Morrissey et al., 1995; Trachtenberg and Thompson, 1996). Direct axonal contact has long been known to induce DNA synthesis in Schwann cell precursors. Antibodies against neuregulin or erbB2 inhibit this axon-driven proliferation, indicating that the neuronal signal is given by an axon-bound isoform of neuregulin (Morrissey et al., 1995). Moreover, experimental evidence indicates that neuregulin provides an important signal for maturation of the neuromuscular junction (Falls et al., 1993; Jo et al., 1995) and for alveolar differentiation of the mammary gland epithelium (Peles et al., 1992; Yang et al., 1995). The strong expression of type II and type III neuregulins observed in the central nervous system of the perinatal animal implies additional functions in the development and/or maintenance of the brain or spinal cord. Neuregulin has been reported to stimulate growth of immature oligodendrocytes and to inhibit their differentiation (Canoll et al., 1996). Functions in development of neurons have been suggested by the analysis of mice that lack an *erbB4* gene, and by the observation that neuregulin affects outgrowth and survival of retinal neurons in culture (Gassmann et al., 1995; Bermingham et al., 1996). Many of these potential roles of neuregulins are as yet not accessible to genetic analysis in the mouse, since all existing *neuregulin* mutations cause defects in heart development and death in utero on E10.5. Our data indicate that heart malformation is caused by a lack of type I neuregulin. Isoform-specific mutations that allow the production of type I neuregulin might thus allow the genetic analysis of additional roles of this versatile growth and differentiation factor.

Neuregulin and cranial ganglia formation

In the cranial portion of the embryo, cells derived from various lineages express type I neuregulin. We detect active β-galactosidase in cells with undifferentiated mesenchymal appearance already on E6.5 and E7, indicating that cells of mesodermal origin express type I neuregulin. Also, neural crest-derived structures like the branchial arch cartilage express neuregulin. Moreover, type I neuregulin is also found in the trigeminal ganglion, which derives from the neural crest and the placode. Type I expressing cells of the trigeminal ganglion appear to be placodal in origin. Ganglion structures least affected by the homozygous *neuregulin$^{\Delta EGF\text{-}lacZ}$* mutation are placodally derived and mutant trigeminal ganglia consist almost exclusively of neuregulin type I-expressing cells, whereas non-expressing cells are lost (Meyer and Birchmeier, 1995). Therefore, the signal essential for survival of neural crest-derived neurons in cranial ganglia might be given by placodal cells. However, cells of other lineage(s) might also contribute to the production of the essential neuregulin signal that drives development of crest-derived neurons in cranial ganglia.

The question arises of why the generation of type III neuregulin in cells of cranial ganglia of *neuregulin^{Ig}* mutant animals suffices to rescue developing Schwann cells, but not neural crest-derived neurons. It should be noted that the temporal window of type I and type III neuregulin expression in cranial structures is overlapping, but type I expression precedes expression of type III. Therefore, the loss of neurons in *neuregulin^{Ig}* mutant animals might reflect a requirement for neuregulin at an early developmental stage, when only type I neuregulin is produced in the embryo. Indeed, the trigeminal ganglia of neuregulin mutant animals appear abnormal already on E9.5, i.e. before type III neuregulin is expressed (Meyer and Birchmeier, 1995). The fact that Schwann cells develop in cranial ganglia of *neuregulin^{Ig}* mutant animals demonstrates that this early type I neuregulin signal is required only for the neural crest-derived neuronal precursors, and not for a common precursor of Schwann cells and neurons. Interestingly, a membrane bound type III neuregulin was recently identified which is an abundant variant produced by neurons; this variant might correspond to the axon-bound neuregulin observed previously (Marc Marchionni, personal communication). Type I and type III neuregulin isoforms might be soluble and membrane bound factors, respectively, and type III neuregulin might only be available for cells that associate closely with axonal membranes, i.e. Schwann cells. Thus differences in expression patterns and, potentially, in subcellular localization might account for the isoform specific functions observed in vivo.

Genetic analyses in the mouse have demonstrated that tyrosine kinase receptors (c-*kit*, c-*ret* and *erbB3*/*erbB2*) play important roles in development of neural crest cells. c-*Kit* is essential for survival and growth of melanocytes (Steel et al., 1992; Cable et al., 1995; Wehrle and Weston, 1995), c-*ret* is important for survival and growth of cells that form enteric and sympathetic ganglia (Schuchardt et al., 1994; Durbec et al., 1996). A common theme in these processes is that the receptors receive a signal that is provided by the target site of neural crest cell migration. Thus, the skin produces steel, the ligand of c-kit, to support melanocytes (Keshet et al., 1991; Besmer et al., 1993; Wehrle and Weston, 1995), enteric mesenchyme provides GDNF, the ligand of c-ret, which is essential for the developing cells of the enteric nervous system (Springer et al., 1994; Trupp et al., 1995; Sanchez et al., 1996; Suvanto et al., 1996; Trupp et al., 1996). These signals ensure growth and survival of the neural crest-derived precursor cells that reached their appropriate position in the embryo. Type I neuregulin, the

ligand for erbB3/erbB2, is required for the development of neural crest-derived neurons in cranial ganglia and is produced at sites of cranial ganglia formation. During cranial ganglia formation, neuregulin might similarly act in a paracrine manner to ensure survival and formation of neural crest-derived sensory neurons.

Functions of the erbB3 and erbB4 receptors

Neuregulin can bind directly to the erbB4 or erbB3 receptors. Since erbB4 and erbB3 are mostly expressed by distinct cell types during development, the receptors take over non-redundant functions in vivo. For instance, a prominent site of expression of erbB4 in the developing embryo is the myocardium where erbB3 is not expressed and which requires this receptor and neuregulin in order to form ventricular trabecules (Gassmann et al., 1995; Meyer and Birchmeier, 1995). Similarly, erbB3, but not erbB4, is expressed in neural crest and Schwann cells; crest-derived neurons of cranial ganglia as well as Schwann cells require neuregulin and erbB3 for their appropriate development (Meyer and Birchmeier, 1995; D. Riethmacher and C.B., unpublished data). In all theses cases, erbB2 is an essential co-receptor for erbB3 and erbB4. However, although complementary expression patterns of the erbB3 and erbB4 receptors are observed frequently, a few cell types (skeletal muscle and epithelia) co-express both receptors. It is thus conceivable that in such cells the two receptors take over redundant functions. Signaling specificity, although little understood on a molecular level, has been observed for tyrosine kinase receptors. The three receptors, erbB2, erbB3 and erbB4, which participate and cooperate in the transmission of the signals given by neuregulin, can each interact with a distinct subset of substrates. Therefore, signaling cascades and cellular responses induced by the various neuregulin receptor combinations might differ in a subtle manner.

We want to thank Marc Marchionni (Cambridge NeuroScience Inc.) and Nancy Rattner (University of Cincinnati) for the gift of a GGF-specific probe and for critical discussions, Lino Tessarollo (NCI Frederick) and L. Reichardt (UCSF) for a rat p75 cDNA probe as well as Walter Birchmeier and Stefan Britsch (MDC, Berlin) for reading the manuscript. T. Y. and A. G. were supported by grants from Okayama University, Japan, and the European Community (TMR program), respectively. The work was also supported by grants from the Deutsche Forschungsgemeinschaft and Bundesministerium fuer Bildung und Forschung to C. B.

REFERENCES

Beerli, R. R., Graus-Porta, D., Woods-Cook, K., Chen, X., Yarden, Y. and Hynes, N. E. (1995). Neu differentiation factor activation of erbB3 and erbB4 is cell specific and displays a differential requirement for erbB2. *Mol. Cell. Biol.* **15**, 6496-6503.

Bermingham, M. O., McCabe K. L. and Reh, T. A. (1996). Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina. *Development* **122**, 1427-38.

Besmer, P., Manova K., Duttlinger R., Huang E. J., Packer A., Gyssler C. and Bachvarova R. F. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Development* **Supplement**, 125-37.

Buck, C. R., Martinez, H. J., Chao, M. V. and Black, I. B. (1988). Differential expression of the nerve growth factor receptor gene in multiple brain areas. *Brain Res. Dev. Brain Res.* **44**, 259-68.

Cable, J., Jackson I. J. and Steel K. P. (1995). Mutations at the W locus affect survival of neural crest derived melanocytes in the mouse. *Mech. Dev.* **50**, 139-50.

Canoll, P. D., Musacchio, J. M., Hardy, R., Reynolds, R., Marchionni M. A. and Salzer, J. L. (1996). GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. *Neuron* **17**, 229-243.

Carraway, K. III and Burden S. J. (1995). Neuregulins and their receptors. *Curr. Opin. Neurobiol.* **5**, 606-612.

Carraway, K. III and Cantley, L. C. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8.

Carraway, K. III, Sliwkowski, M. X., Akita, R., Platko, J. Y., Guy, P. M., Nuijens, A., Diamonti, A. R., Vandlen, R. L., Cantley L. C. and Cerione, R. A. (1994). The erbB3 gene product is a receptor for heregulin. *J. Biol. Chem.* **269**, 14303-14306.

Chen, M. S., Berminghammcdonogh, O., Danehy, F. T., Nolan, C., Scherer, S. S., Lucas, J., Gwynne, D. and Marchionni, M. A. (1994). Expression of multiple neuregulin transcripts in postnatal rat brains. *J. Comp. Neurol.* **349**, 389-400.

Corfas, G., Rosen, K. M., Aratake, H., Krauss, R. and Fischbach, G. D. (1995). Differential expression of ARIA isoforms in the rat brain. *Neuron* **14**, 103-115.

D'Amico, M. A. and Noden, D. M. (1983). Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. *Am. J. Anat.* **166**, 445-468.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-596.

Durbec, P. L., Larsson, B. L., Schuchardt, A., Costantini, F. and Pachnis, Y. (1996). Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development* **122**, 349-358.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72**, 801-815.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the erbb4 neuregulin receptor. *Nature* **378**, 390-394.

Hamburger, V. (1961). Experimental analysis of the dual origin of the trigeminal ganglion in the chick embryo. *J. Exp. Zool.* **148**, 91-124.

Ho, W. H., Armanini, M. P., Nuijens, A., Phillips, H. S. and Osheroff, P. L. (1995). Sensory and motor neuron derived factor: a novel heregulin variant highly expressed in sensory and motor neurons. *J. Biol.Chem.* **270**, 14523-14526.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-10.

Jo, S. A., Zhu, X. J., Marchionni, M. A. and Burden, S. J. (1995). Neuregulins are concentrated at nerve-muscle synapses and activate ach-receptor gene expression. *Nature* **373**, 158-161.

Keshet, E., Lyman, S. D., Williams, D. E., Anderson, D. M., Jenkins, N. A., Copeland, N. G. and Parada, L. F. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J.* **10**, 2425-35.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 4833-8.

Lai, C. and Lemke, G. (1991). An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. *Neuron* **6**, 691-704.

LeDouarin, N. M. (1982). *The Neural Crest.* Cambridge, Cambridge University Press.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbb2 in neural and cardiac development. *Nature* **378**, 394-398.

Lemke, G. (1996). Neuregulins in development. *Molecular And Cellular Neuroscience* **7**, 247-262.

Lemke, G. and Chao, M. (1988). Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* **102**, 499-504.

Marchionni, M. A. (1995). Cell cell signalling: neu tack on neuregulin. *Nature* **378**, 334-335.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham, M. O.,

Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Marikovsky, M., Lavi, S., Pinkas, K. R., Karunagaran, D., Liu, N., Wen, D. and Yarden, Y. (1995). ErbB-3 mediates differential mitogenic effects of NDF/heregulin isoforms on mouse keratinocytes. *Oncogene* **10**, 1403-1411.

Meyer, D. and Birchmeier, C. (1994). Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. *Proc. Natl. Acad. Sci. USA* **91**, 1064-1068.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Morrissey, T. K., Levi, A. D., Nuijens, A., Sliwkowski, M. X. and Bunge, R. P. (1995). Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. *Proc. Natl. Acad. Sci. USA* **92**, 1431-1435.

Orr-Urtreger, A., Trakhtenbrot, L., Ben, L. R., en, D., Rechavi, G., Lonai, P. and Yarden, Y. (1993). Neural expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. *Proc. Natl. Acad. Sci. USA* **90**, 1867-1871.

Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B. and Yarden, Y. (1992). Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* **69**, 205-16.

Peles, E., Ben, L. R., Tzahar, E., Liu, N., Wen, D. and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. *EMBO J.* **12**, 961-971.

Peles, E. and Y. Yarden (1993). Neu and its ligands: from an oncogene to neural factors. *BioEssays* **15**, 815-824.

Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc. Natl. Acad. Sci. USA* **87**, 4905-4909.

Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, J. M. and Buckley, S. (1993). Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature* **366**, 473-475.

Sanchez, M. P., Silos, S. I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-3.

Schuchardt, A., D'Agati, V., Larsson, B. L., Costantini. F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-383.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P. and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* **77**, 349-360.

Skarnes, W. C., Moss, J. E., Hurtley, S. M. and Beddington, R. S. (1995). Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl. Acad. Sci. USA* **92**, 6592-6596.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, K. III (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* **269**, 14661-14665.

Sonnenberg, E., Godecke, A., Walter, B., Bladt, F. and Birchmeier, C. (1991). Transient and locally restricted expression of the ros1 protooncogene during mouse development. *EMBO J.* **10**, 3693-3702.

Springer, J. E., Mu, X., Bergmann, L. W. and Trojanowski, J. Q. (1994). Expression of GDNF mRNA in rat and human nervous tissue. *Exp. Neurol.* **127**, 167-170.

Steel, K. P., Davidson, D. R. and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-1119.

Suvanto, P., Hiltunen, J. O., Arumae, U., Moshnyakov, M., Sariola, H., Sainio, K. and Saarma, M. (1996). Localization of glial cell line derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. *European Journal Of Neuroscience.* **8**, 816-822.

Trachtenberg, J. T. and Thompson, J. W. (1996). Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379**, 174-177.

Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A. S., Sieber, B. A., Grigoriou, M., Kilkenny, C., Salazar, G. E., Pachnis, V., Arumae, U., Sariola, H., Saarma, M. and Ibanez, C. F. (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* **381**, 785-788.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E. and Ibanez, C. F. (1995). Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell. Biol*. **130**, 137-148.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N. L., Yayon, A., Wen, D. Z. and Yarden, Y. (1994). Erbb-3 and erbb-4 function as the respective low and high affinity receptors of all neu differentiation factor heregulin isoforms. *J. Biol. Chem.* **269**, 25226-25233.

Wang, H. U. and Anderson, D. J. (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-396.

Wehrle, H. B. and Weston, J. A. (1995). Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development* **121**, 731-742.

Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., et. al. (1992). Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* **69**, 559-572.

Wen, D., Suggs, S. V., Karunagaran, D., Liu, N., Cupples, R. L., Luo, Y., Janssen, A. M., Ben, B. N., Trollinger, D. B., Jacobsen, V. L. et. al. (1994). Structural and functional aspects of the multiplicity of Neu differentiation factors. *Mol. Cell. Biol.* **14**, 1909-1919.

Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, M., Birchmeier, C. and Birchmeier, W. (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogensis and differentiation of the mammary gland. *J. Cell. Biol.* **131**, 1-12.

(Accepted 26 June 1997)