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Neuregulins: functions, forms, and signaling strategies

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Abstract

The neuregulins (NRGs) are cell-cell signaling proteins that are ligands for receptor tyrosine kinases of the ErbB family. The neuregulin family of genes has four members: *NRG1*, *NRG2*, *NRG3*, and *NRG4*. Relatively little is known about the biological functions of the NRG2, 3, and 4 proteins, and they are considered in this review only briefly. The NRG1 proteins play essential roles in the nervous system, heart, and breast. There is also evidence for involvement of NRG signaling in the development and function of several other organ systems, and in human disease, including the pathogenesis of schizophrenia and breast cancer. There are many NRG1 isoforms, raising the question "Why so many neuregulins?" Study of mice with targeted mutations ("knockout mice") has demonstrated that isoforms differing in their N-terminal region or in their epidermal growth factor (EGF)-like domain differ in their in vivo functions. These differences in function might arise because of differences in expression pattern or might reflect differences in intrinsic biological characteristics. While differences in expression pattern certainly contribute to the observed differences in in vivo functions, there are also marked differences in intrinsic characteristics that may tailor isoforms for specific signaling requirements, a theme that will be emphasized in this review.

Keywords: Neuregulin; Acetylcholine receptor-inducing activity; Glial growth factor; Heregulin; Neu differentiation factor; Sensory and motor neuron-derived factor; ErbB receptor tyrosine kinase; Schizophrenia; Neuromuscular synapse; Cell-cell signaling proteins; Juxtacrine signaling; Paracrine signaling; Transmembrane ligands; Proteolytic process; Shredding

The discovery of neuregulins (NRGs); NRG family genes; the focus of this review

Neuregulins (NRGs) are signaling proteins that mediate cell-cell interactions in the nervous system, heart, breast, and other organ systems. "Forward" signaling by NRGs—i.e., signaling from a NRG-producing cell to a NRG-responsive cell—involves binding of NRG to the extracellular domain of the receptor tyrosine kinases ErbB3 or ErbB4, which leads to formation of ErbB homo- or heterodimers (often including ErbB2), which in turn activates intracellular signaling pathways leading to cellular responses that include stimulation or inhibition of proliferation, apoptosis (programmed cell death), migration, differentiation, and adhesion [1].

The first identifications of NRGs were reported in 1992–1993 by four groups. Two of these groups sought a ligand for the oncogene ErbB2 (a.k.a. neu, HER2) [2–4]; the third sought a factor that stimulated the proliferation of Schwann cells [5,6], and the fourth sought a factor that stimulated the synthesis by muscle of receptors for acetylcholine, the major neurotransmitter at developing neuromuscular synapses [7]. The neuregulin proteins isolated by each of these groups are encoded by the gene that would now be referred to as NRG1. It should be noted that though one approach leading to the identification of NRGs was a search for ErbB2 ligands, in fact, it appears that NRG proteins interact with ErbB2 only after binding ErbB3 or ErbB4 [1].

Subsequent to the identification of the *NRG1* gene, three other genes encoding related proteins were discovered. These "other" NRGs are referred to as NRG2 (a.k.a. Don-1, NTAK [8–11]), NRG3 [12], and NRG4 [13]. The NRG1 proteins effectively bind to both ErbB3 and ErbB4; the protein products of these other NRG genes effectively bind one or the other or both of these ErbBs [1,14,15]. Very little is yet known about the functions of the NRG2, 3, and 4

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proteins. One intriguing recent finding is evidence of NRG4 involvement in the differentiation of the somatostatin-expressing delta cells of pancreatic islets of islets [16]. Buonanno and Fischbach [17] compare the structure and expression patterns of NRG2, 3, and 4 proteins to NRG1 proteins and discuss the intriguing discovery that NRG2 activation of a specific ErbB receptor combination (i.e., ErbB4 homodimers) can elicit different patterns of receptor phosphorylation and downstream consequences than activation of the same receptor combination in the same cell type by NRG1 [18,19].

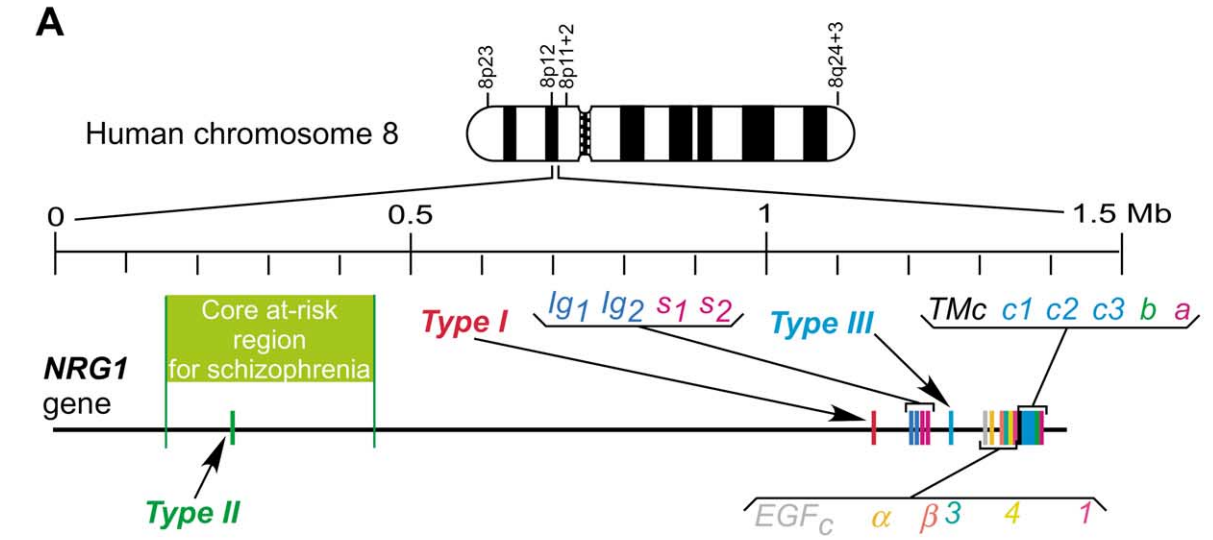
This review will focus on the NRG1 proteins, and unless explicitly indicated otherwise, the terms "neuregulin" and "NRG" here refer to NRG1 proteins. Since the discovery of NRGs 10 years ago, the field has grown rapidly. A search of Pub Med for "neuregulin" or various names by which these proteins have also been known (see below) in early December 2002 returned a list of over 800 publications. This rapid growth in the literature attests to the importance of NRGs, but also means that that this review cannot be in any sense comprehensive. Thus, I apologize in advance for the many important papers not referenced and for interesting areas of NRG research omitted or mentioned only in passing. A number of excellent reviews of NRGs and NRG-mediated cell-cell interactions have appeared over the years. Selected reviews published in the last 6 years include those focused specifically on NRGs [17,20–25], as well as reviews of neuromuscular synapse development [26–28], neuron-glial interactions [24,29–33] and cell interactions regulating heart development and function [169]. Companion reviews in this issue discuss the NRG receptors ErbB2, ErbB3, and ErbB4 and intracellular signaling pathways activated by these receptors (see reviews in this issue by Yarden, Carpenter, and Wiley; see also [1,17]). Other companion reviews in this issue describe the roles of ErbB family receptors and ligands in breast development, in cancer, and as therapeutic targets (reviews by Stern, Hynes, Arteaga, Fry, and Maihle).

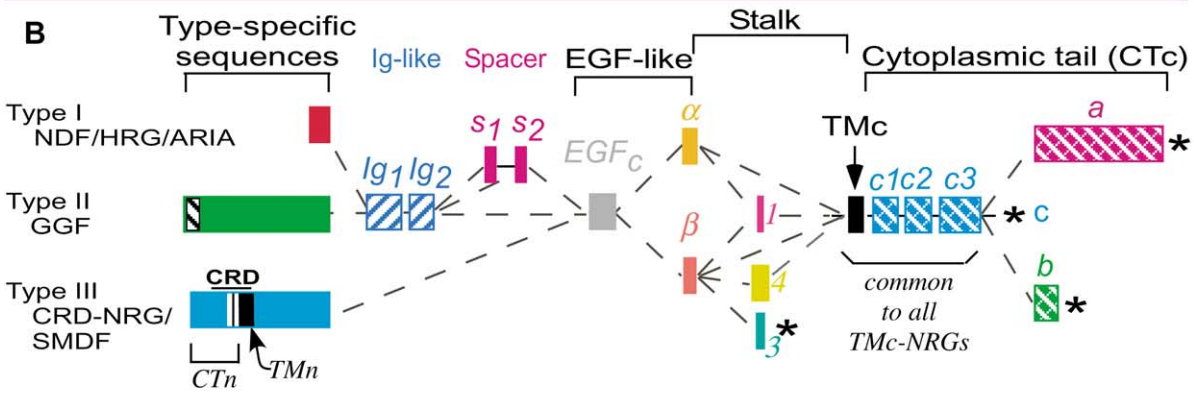
While I will begin with an overview of NRG biology, the availability of these other reviews allows me to emphasize recent literature and to focus on the normal biological functions of the NRG1 proteins during development and in the adult, evidence for the involvement of NRG1 signaling in neuropathology (other than cancer), and mechanisms regulating NRG signal production. Readers of the companion reviews on the EGFR and its ligands (see reviews by Coffey and Burgess) and the ErbB receptor/ligand homologues in invertebrates (see reviews by Shilo and Sternberg) will note both marked similarities and striking differences between the biology of NRG1 proteins and these related signaling systems of vertebrates and invertebrates.

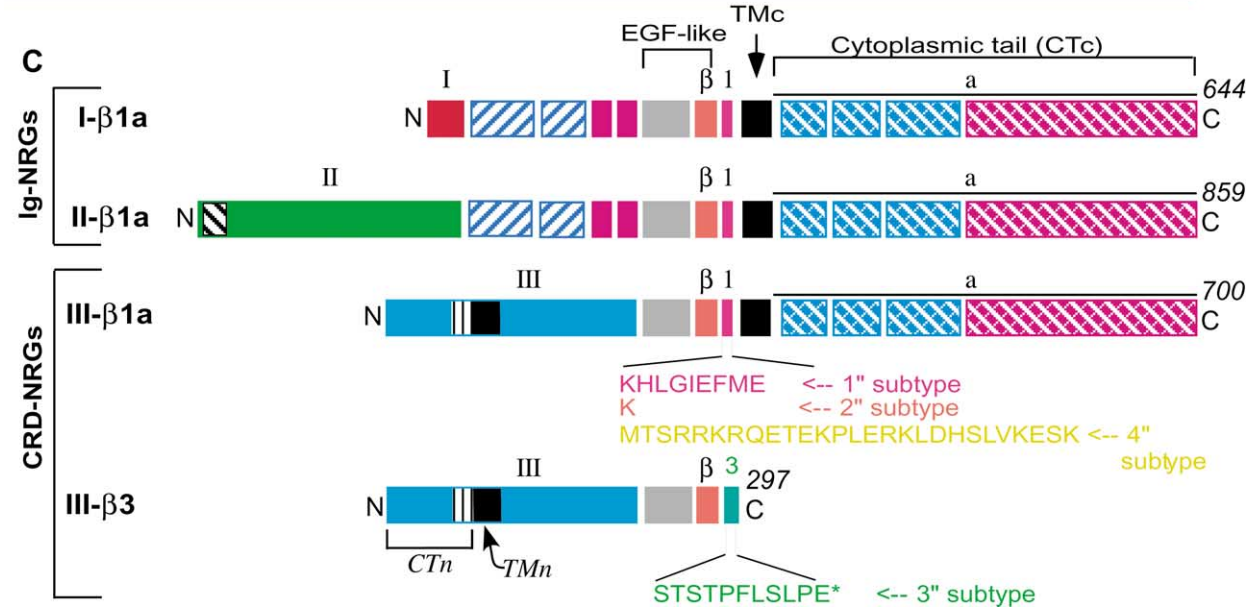
The NRG1 gene; NRG1 isoforms and nomenclature

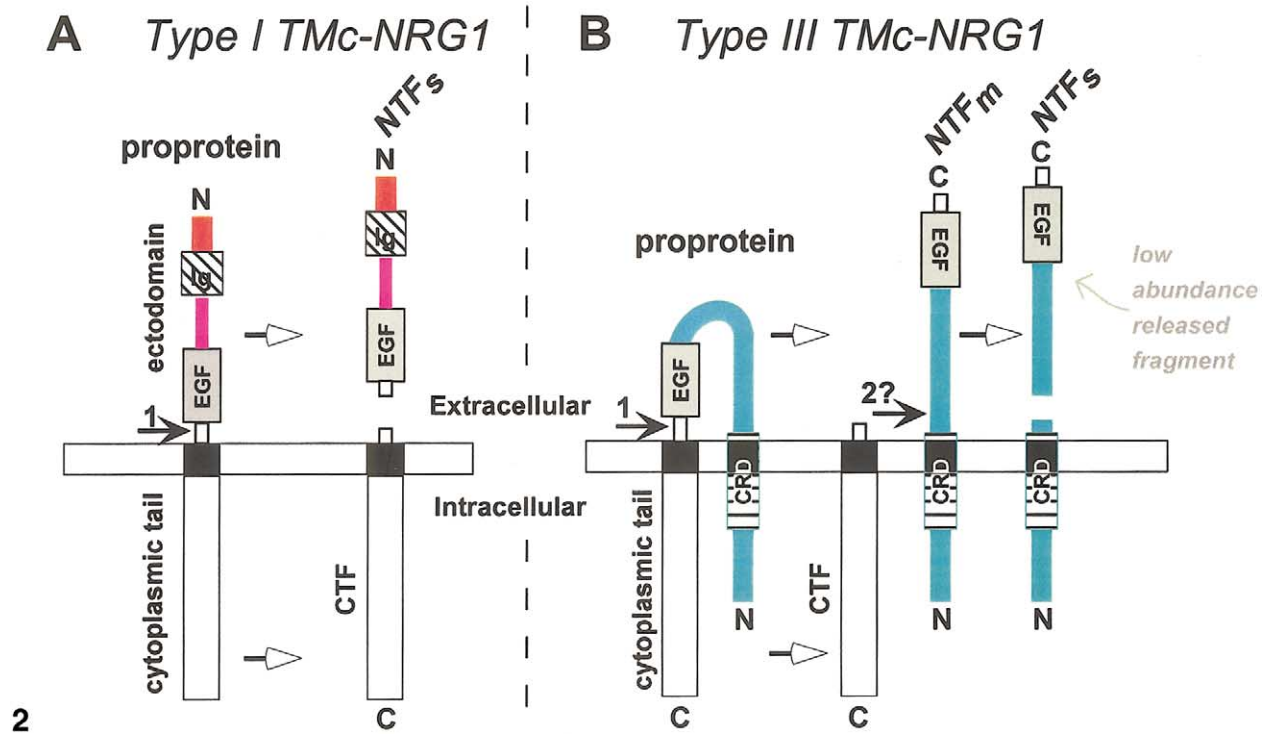
An important recent advance is the sequencing and assembly of the entire human NRG1 gene (Fig. 1A, [34]). The gene is ≈1.4 megabases long (≈1/2000th of the genome); less than 0.3% of this span encodes protein. As a consequence of rich alternative splicing and multiple promoters, at least 15 different NRG isoforms are produced from the single *NRG1* gene (Fig. 1B and C [17,20]). The three structural characteristics we know to importantly differentiate isoforms with respect to in vivo functions and cell biological properties are the type of EGF-like domain (α or β), the N-terminal sequence (type I, II, or III), and whether the isoform is initially synthesized as a transmembrane or non-membrane protein; the import of these differences will be discussed below. The EGF-like domain contained in all bioactive NRG isoforms is alone sufficient for activation of ErbB receptor-tyrosine kinases (see [17] for comparison of NRG1 EGF-like domain sequences to the EGF-like domain in NRG2, 3, and 4 and other EGF family members). Together the types I and II NRGs are sometimes referred to as "Ig-NRGs," and the type III NRGs are sometimes referred to as "CRD-NRGs." The names first used in the literature to refer to various NRG isoforms—acetylcholine receptor-inducing activity (ARIA [7]), glial growth factor (GGF [5,6]),

Fig. 1. → NRG1 gene and isoform structure. (A) Human *NRG1* gene structure (Genbank accession no. BK000383). The NRG1 gene is on the short arm of chromosome 8. On the expanded illustration of this region, the position of each exon included in reported NRG1 isoforms is indicated by a vertical line. Lines descending along the edge of the green box delineate the boundaries of the core at-risk haplotype for schizophrenia. Only the exon encoding the type II-specific N-terminal region lies within these bounds. *Exon naming:* Exons are named here for the structural region of the NRG1 protein they encode. Abbreviations used closely correspond to names of NRG protein structural regions indicated in panel B. EGFc refers to the exon encoding the portion of the EGF-like domain sequence shared by NRGs with an α-type and NRGs with a β-type EGF-like domain. The exon labeled TMc also includes adjacent extracellular juxtamembrane sequence and cytoplasmic tail sequence. (B) Illustration of NRG "coding segments." Isoforms differ in their coding segment composition due to initiation of transcription from different *NRG1* gene promoters and alternative splicing. The EGF-like domain alone is sufficient for high potency activation of the cognate ErbB receptor tyrosine kinases. Available evidence indicates that the NRGs most commonly expressed in the nervous system are transmembrane NRGs with a β-type EGF-like domain and the 374-amino acid a-type tail. Not all potential combinations of coding segments have been reported. CRD = cysteine-rich domain; EGF = epidermal growth factor-like domain; Ig = immunoglobulin-like domain; CTc and TMc = cytoplasmic tail and TM domain C-terminal of the EGF-like domain; CTn and TMn = cytoplasmic tail and TM domain N-terminal of the EGF-like domain. Only type III NRGs have the CTn and TMn. * = stop codon. (C) Structural regions of the I-β1a, II-β1a, III-β1a, and III-β3 proproteins. The I-β1a, II-β1a, and III-β1a isoforms differ only in their N-terminal region; their sequence is identical from the EGF-like domain through the carboxy-terminus. The sequence of III-β1a and III-β2a is identical except that in III-β2a the last eight amino acids of the "1" subtype are absent (illustrated under III-β1a sequence). The sequence of III-β1a and III-β3 is identical from the N-terminus to just beyond the final cysteine of the EGF-like domain. Hydrophobic regions that serve as transmembrane domains are indicated by black boxes. A hydrophobic sequence in the type II N-terminal region is indicated by a hatched box. This is believed to serve as a noncleaved internal signal sequence but not as a transmembrane domain.

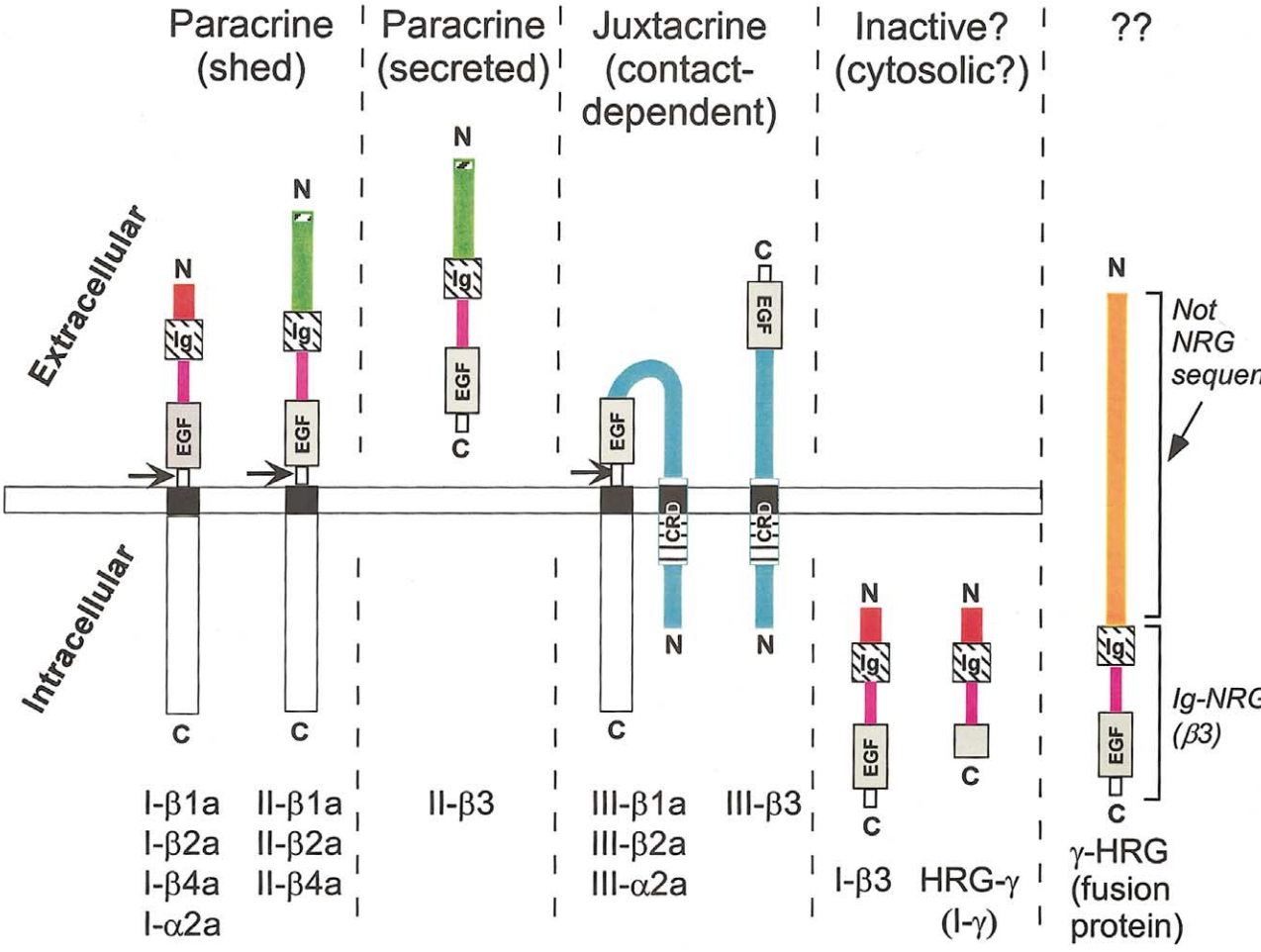

A
Human chromosome 8
8p23
8p12
8p11+2
8q24+3
0
0.5
1
1.5 Mb
Core at-risk region for schizophrenia
NRG1 gene
Type I
Ig1 Ig2 s1 s2
Type III
TMc c1 c2 c3 b a
Type II
EGFc α β3 4 1


B
Type-specific sequences
Ig-like
Spacer
EGF-like
Stalk
Cytoplasmic tail (CTc)
Type I NDF/HRG/ARIA
Type II GGF
Type III CRD-NRG/ SMDF
CRD
CTn
TMn
Ig1 Ig2
s1 s2
EGFc
α
β
1
4
3*
TMc
c1c2 c3
a
b
c
common to all TMc-NRGs


C
Ig-NRGs
CRD-NRGs
I-β1a
II-β1a
III-β1a
III-β3
EGF-like
TMc
Cytoplasmic tail (CTc)
644
859
700
297
KHLGIEFME <-- 1" subtype
K <-- 2" subtype
MTSRRKRQETEKPLERKLDHSLVKESK <-- 4" subtype
STSTPFLSLPE* <-- 3" subtype


A
Type I TMc-NRG1
proprotein
NTFs
N
ectodomain
Ig
EGF
1
Extracellular
Intracellular
cytoplasmic tail
CTF
C
B
Type III TMc-NRG1
NTFm
NTFs
C
EGF
proprotein
low abundance released fragment
1
2?
CRD
N
cytoplasmic tail
CTF
C

2


Paracrine (shed)
Paracrine (secreted)
Juxtacrine (contact-dependent)
Inactive? (cytosolic?)
??
Extracellular
Intracellular
N
Ig
EGF
C
CRD
Not NRG sequence
Ig-NRG (β3)
I-β1a
I-β2a
I-β4a
I-α2a
II-β1a
II-β2a
II-β4a
II-β3
III-β1a
III-β2a
III-α2a
III-β3
I-β3
HRG-γ (I-γ)
γ-HRG (fusion protein)

3

heregulin (HRG [2]), neu differentiation factor (NDF [3,4]), and sensory and motor neuron-derived factor (SMDF [35]), —cannot be taken to indicate specific biological functions of the isoforms to which these names have been applied. For example, it now seems likely that the major NRG isoforms that act as "glial growth factors" in vivo are type III NRGs, not the type II NRGs originally called glial growth factor (GGF).

NRG1 signaling in health; isoforms differing in their N-terminal region or EGF-like domain differ in their in vivo functions

Without NRGs life is not possible. However, even I—a confirmed neuregulin fanatic—was surprised as I surveyed the literature in preparation for writing this review, at how pervasive NRG signaling appears to be (Table 1). While I will here introduce the in vivo functions of NRGs by describing the dramatic phenotypes of the knockouts, it must be emphasized that a number of other experimental approaches have made important contributions to our current understanding of NRG functions, and it seems likely that many NRG functions remain to be discovered.

Studies of mice with targeted mutations of the *NRG1* gene have been very valuable in elucidating functions of NRG1 proteins [34,36–47]. Analysis of pan-NRG1 knockout (KO) mice (mice in which all NRG isoforms are unable to bind to and activate ErbB receptors due to disruption of the EGF-like domain) revealed an essential role of NRGs in cardiac morphogenesis [36], a role unsuspected from previous studies of NRG bioactivities. Due to the defect in cardiogenesis, these mice die midway through embryogenesis (E10.5), the time at which mouse embryos switch from dependence on the maternal circulation to dependence on their own circulation. The pan-NRG1 KO mice also have a severe reduction in several neural crest-derived cell populations including Schwann cells, the glia of the peripheral nervous system, which—among other things—form the myelin sheaths of peripheral nerves; neural crest-derived cranial sensory neurons; and sympathetic neurons [39,40]. Having a knockout with a severe phenotype is both a boon and a bane: a boon because it provides comforting reassurance that the gene of interest has essential roles and a bane because death or early developmental disruptions in the mutants preclude analysis of later developmental events. Such is the case with analysis of nervous system development in the pan-NRG1 knockout mice: at E10.5 the development of the nervous system is only beginning to unfold; and the role of NRGs in processes such as neuromuscular synaptogenesis, which begins around E14, and the development of oligodendrocytes, the cells that myelinate axons in the central nervous system, is inaccessible for direct in vivo examination in these mice. In some cases, clever strategies can allow this limitation to be partially circumvented. Thus, through analysis of spinal cord slices harvested from E9.5 pan-NRG1 embryos and maintained in organ culture for up to 11 days, a strong case has been made for an essential role of NRGs in oligodendrocyte lineage development [48].

Mice with targeted mutations that inactivate only certain classes of NRG isoforms have revealed differential in vivo functions of NRG proteins (Table 2). *Ig-NRG1*$^{-/-}$ mice: Mice with all Ig-NRG isoforms inactivated (types I and II NRGs inactivated)—but in which CRD-NRG (Type III NRG) production is presumably normal—die at E10.5 and have defects in cardiac, cranial sensory neuron, and sympathetic development similar to those of the pan-NRG knockouts [37,39,40]. However, unlike the pan-NRG KOs, the Ig-NRG KO mice have normal development of Schwann cell precursors [39]. *CRD-NRG1*$^{-/-}$ *mice:* In contrast to the pan- and Ig-NRG KO mice, mice with all CRD-NRG isoforms (type III NRGs) inactivated—but with normal expression of Ig-NRGs—do not have defects in heart development [42]. The CRD-NRG KO embryos survive to birth. They die at birth because they cannot breathe;

Fig. 2. Membrane orientation and proteolytic processing proposed for the NRG I-β1a isoform (A) and III-β1a isoform (B) based on studies in transfected fibroblastic cell lines. (A) Proteolytic cleavage of the I-β1a proprotein in the "stalk" region (arrow no. 1) produces an N-terminal fragment (NTF) containing the bioactive EGF-like domain and a C-terminal fragment (CTF), also referred to as the "a-tail remnant." The NTF is efficiently released into the medium. The protease(s) catalyzing stalk cleavage—at least in fibroblasts—is likely to be a metalloprotease. All Type I and Type II proproteins with a transmembrane domain are expected to have similar topology and processing (see Fig. 3). (B) Available evidence indicates that the III-β1a proprotein has two transmembrane domains and that proteolytic cleavage in the stalk region (arrow no. 1) produces a transmembrane N-terminal fragment (NTF_m) that accumulates at the cell surface. Cleavage of the III-β1a NTF_m near the membrane (arrow no. 2) can release a fragment (NTF_s) containing the EGF-like domain into the medium, but for NRGs expressed in fibroblasts, the amount of this released Type III NTF_s is very small compared to the amount of released Type I NTF_s. All Type III proproteins with a transmembrane domain C-terminal of the EGF-like domain are expected to have topology and processing similar to that illustrated here for III-β1a (see Fig. 3).

Fig. 3. Proposed topology and mode of presentation to receptor for selected NRG1 proprotein isoforms. The data and reasoning supporting the assigned topology and mode of presentation (paracrine, shed; paracrine, secreted; juxtacrine) for the types I, II, and III isoforms are described in the text. The proteins encoded by two other mRNA sequences are illustrated to facilitate their comparison with the full-length NRGs shown. HRG-γ is a truncated Type I sequence. It is unlikely to be bioactive for two reasons: first, the EGF-like domain, which is necessary for activating ErbBs, is incomplete; and second, since it is a truncated version of I-β3, it is unlikely to be released. The rightmost diagram illustrates a protein named γ-HRG ($\neq$HRG-γ). This protein is encoded by transcripts produced by the breast cancer cell line MDA-MB-175 [166]. It has now been shown that γ-HRG is a fusion protein with N-terminal sequence from the human homologue of transcription factor DOC-4 [167]. This transcription factor is a member of the Oz/ten M family. On the basis of sequence similarity to the N-terminal portion of γ-HRG, some sequences encoding Ten-m/Odz family members have been erroneously labeled as NRGs or "NRG-like." There is as yet no evidence for a physiological role of the HRG-γ sequences or γ-HRG [168], and they are not further discussed in the text.

Table 1
Selected proposed functions of NRGs[a]

Organ/cell type/ structure	Effect	Reference(s)
Nervous system		
Schwann cells	Survival, proliferation, migration, differentiation, myelination	[24,29,31–33]
Oligodendrocytes	Proliferation, survival, differentiation, myelination	[48,129–134,153]
Neuromuscular synapse	Nerve–muscle interaction controlling initial formation, acetylcholine receptor synthesis during development and in the adult, nerve terminal interactions with "terminal Schwann cells"	[17,20,26–28,146]
Muscle spindle	Muscle spindle development (Muscle spindles are muscle length/stretch sensors.)	[172]
Cranial sensory neurons	Initial population of cranial sensory ganglia (ganglia of cranial nerves) with neural-crest derived sensory neurons (However, the initial population of cranial sensory ganglia with placode-derived sensory neurons appears to be unaffected by *NRG1* mutations.)	[36–39,44]
Motor and sensory neurons	Survival (spinal and probably also cranial)	[42]
Peripheral and cranial nerves	Fasciculation (bundling) of axons and/or integrity of nerves	[36–39,42,44]
Sympathetic neurons/adrenal medulla	Migration of sympathetic neuron/adrenal chromaffin precursors to the anlage of sympathetic ganglia/adrenal medullas	[40]
Cerebellum	Production of cerebellar neuron precursors	[38]
Cortical neuron precursors/ cerebellar granule cells	Migration of CNS neuronal precursors along radial glia	[71,72]
Hypothalamus	Hypothalamic control of mammalian female sexual maturation	[135,173]
Parasympathetic	Enteric ganglia development	[136,174,175]
Hippocampus	Inhibition of long-term potentiation (LTP) induction (LTP is a model for studying the neurophysiological basis of learning and memory.)	[97]
Various neurons of CNS and PNS	Regulation of neuronal neurotransmitter receptors (NMDA, GABA, neuronal nicotinic acetylcholine receptors) and other neuronal ion channels	[64,74–76,176]
Heart	Development of ventricular wall trabeculae, AV-septum, and cardiac valves	[36–38,44,137]
Heart	Development of cardiac conduction system	[138]
Heart	Growth, repair, survival of adult cardiomyocytes; response to increased work load	[89–91,139]
Blood vessels	Angiogenesis	[144]
Breast	Breast development during pregnancy and lactation	[45]; Review in this issue by D. Stern
Lung	Development of pulmonary epithelium (autocrine effect?)	[142]
Muscle	Myogenesis (autocrine effect?)	[140]
Muscle	Muscle fiber survival in neonatal period	[141]
Muscle	Glucose uptake	[144]
Gonads	Gonadogenesis	[145,177]
Stomach	Proliferation of gastric epithelium; regulation of parietal and chief cell population size	[178,179]

[a] This list is not intended to be comprehensive, and further investigation will be required to confirm the physiological significance of many of the proposed roles. Some proposed functions have been inferred principally from the effects of exogenously supplied recombinant NRG1, ErbB blockade, or ErbB knock-out; in these cases the physiological signal may actually be NRG2, 3, or 4 or another ErbB ligand. Caveat emptor. Functions proposed solely on the basis of mRNA/protein expression data are not included. Reviews have been cited for proposed functions of NRG1s in Schwann cell and neuromuscular synapse development due to the large body of relevant literature.

they cannot breathe because they do not have functional neuromuscular synapses. Unlike the Ig-NRG knockouts, the CRD-NRG knockouts have a marked reduction in Schwann cell precursors. Other prominent phenotypic characteristics of the CRD-NRG KO mice include degeneration of peripheral and cranial nerves and an ≈50% reduction in the number of spinal motor and sensory neurons. The reduction in motor and sensory neuron number appears to be due to abnormal neuron death, as the initial number of these (postmitotic) neurons is normal. Like the spinal motor and sensory neurons, in the *CRD-NRG1* KO mice, cranial motor and sensory neurons (which together contribute most of the axons that make up the cranial nerves) appear to be reduced in number. However, unlike the *Ig-NRG1* KO mice, both placode- and neural crest-derived cranial sensory neurons are affected. In the *Ig-NRG1* KOs, the reduction in neural crest-derived cranial sensory neurons is due to a defect in initial accumulation of these neurons in the nascent ganglia (perhaps, like the defect in sympathetic neuron accumulation, caused by abnormal neuronal precursor migration?), but in the *CRD-NRG1* KO mice it seems likely that the reduction in cranial sensory neurons results from abnormal death of neurons, and, as for spinal sensory neurons, this increased death is probably a consequence of disruption in signaling between these neurons and their supporting Schwann cells or targets. *$NRG1^{-/-}$ mice:* Mice with a targeted mutation that inactivates all NRG isoforms with an α-type EGF-like domain (NRGαs)—but in which production of isoforms with β-type EGF-like domain (NRGβs) is presumably normal—have not been reported to have abnor-

Table 2
Comparison of NRG1 knockout mice with respect to selected characteristics

Development of:	Genotype and isoforms INactivated				
	NRG1$^{-/-}$ All (Pan-NRG1 KO)	*Ig-NRG1*$^{-/-}$ Ig-NRG1 (Type I and II)	*CRD-NRG1*$^{-/-}$ CRD-NRG1 (Type III)	*NRG1α*$^{-/-}$ NRG1α	*NRG1*$^{\Delta CT/\Delta CT}$ Type I NRG1 (and others?)**
Heart	■	■			■
Schwann cell precursors	■		■		?? (Not described)
Neuromuscular synapses	□		■		□
Breast (during pregnancy)	□	□	□	■	□
Homozygotes die at	E10.5	E10.5	Birth	Normal lifespan	E10.5
Cause of death:	Heart failure	Heart failure	Respiratory (neuromuscular) failure	Old age	Heart failure
References	[36, 38, 39]	[37]	[42]	[45]	[44]

[a] The major neurotransmitter receptor at neuromuscular synapses is the muscle nicotinic acetylcholine receptor. Although neuromuscular synapse development (which begins around E14) cannot be assessed in the Ig-NRG KOs, adult Ig-NRG$^{+/-}$ mice (i.e., heterozygous for the mutation inactivating Ig-NRGs) have a 50% reduction in the number of acetylcholine receptors at neuromuscular synapses [47], indicating that Ig-NRGs do function as an "acetylcholine receptor-inducing activity" (ARIA). This "postsynaptic phenotype"—i.e., the effect on AChRs, which are concentrated in the postsynaptic muscle membrane—is different than the "presynaptic" and "Schwann cell phenotype" of the CRD-NRG KO mice (for review of neuromuscular synapse development and NRG functions at the neuromuscular synapse, see refs. [20,146]).

[b] The ΔCT allele of the *NRG1* gene has an in-frame stop codon within the sequence encoding the cytoplasmic tail. This mutation causes all transmembrane NRG1 proteins produced from this allele to have a cytoplasmic tail length of only 3 amino acids. See text ("A tale of the heart . . . ") for interpretation of the *NRG1*$^{\Delta CT/\Delta CT}$ phenotype.

Black = abnormal; blank = normal; shaded = not accessible for analysis because mice die prior to occurrence of this developmental event. Mice are normally born at embryonic day 21 or 22 (E21 or E22).

malities in nervous system or cardiac development, but have marked defects in breast development [45]. (Aside: In most assays, NRG1s with a β-type EGF-like domain are 10–100 times more potent than NRG1s with an α-type EGF-like domain. It is a puzzle as to why NRG1s with both α- and β-type EGF-like domains exist and why the α-type was selected by evolution for a critical role in breast development.)

Comparison of the phenotypes of the ErbB knockouts [34,38,40,41,43,45,49–57] to the NRG1 knockouts has provided insight into the ErbB receptor combinations mediating early essential actions of NRGs. Furthermore, most all reported characteristics of the various NRG1 knockouts are shared by one or more of the ErbB knockouts and vice versa, which suggests that interactions of NRG2, 3, and 4 with ErbBs are unlikely to play a prominent role in the developmental events that dominate the ErbB KO phenotypes; rather these developmental events are likely to be mediated principally by NRG1-ErbB interactions. There is one clear exception to this generalization; ErbB4 knockout mice have a defect in hindbrain segmentation not seen in the NRG1 knockout mice [49], indicating that a non-NRG1 ligand interacts with ErbB4 to guide this developmental event.

One further note, many of the phenotypic characteristics of the NRG1 and ErbB KO mice center on abnormalities of ErbB expressing cell populations, suggesting that these defects result from disruption of forward signaling (NRG-producing cell signaling to ErbB-expressing cell). However, it has been proposed that NRG-ErbB signaling is bidirectional [58–60], similar to what has been demonstrated for Eph-Ephrin signaling (see [61] and references therein). In this model, it is proposed that there is not only the conventional "forward signaling" from NRG-producing cells to ErbB-expressing cells, but that there is also reverse signaling (or "back-signaling") from ErbB-expressing cells to NRG-expressing cells. In the latter case, NRG would serve as the receptor and ErbB the ligand. Some phenotypic characteristics of the NRG1 and ErbB1 knockouts involve NRG-producing cells, raising the possibility that these defects result from disruption of NRG → ErbB reverse signaling. For example, motor neurons, which produce NRGs and which communicate with ErbB expressing Schwann and skeletal muscle cells, are reduced in number in the CRD-NRG KO mice compared to wild-type mice. This might be due to interruption of ErbB → NRG reverse signaling, though alternatively, this abnormality could also result from interruption of forward signaling secondarily disrupting a separate "reciprocal" signaling pathway back to motor neurons from Schwann cells and/or muscle. While the hypothesis of NRG1-ErbB reverse signaling is very attractive, definitive evidence that this occurs has yet to be published.

NRG1 signaling in disease: evidence for involvement in pathophysiology and potential therapeutic uses

The many functions of neuregulins revealed through knockout and other studies (Tables 1 and 2) attest to the importance of neuregulin signaling during development and in the adult. Are disorders of neuregulin signaling involved

Table 3
Diseases/injuries in which the pathophysiology may involve perturbations in NRG1 signaling and/or in which NRG1s may be of therapeutic use[a]

Organ/organ system	Effect	Type of evidence with references
Nervous system	Schizophrenia	[34,62,63,66]
Nervous	Multiple sclerosis (pathology and treatment)	[147–153]
Nervous	Promotion of neural regeneration/proliferation of olfactory ensheathing glia for therapeutic use in neural regeneration	[154–157]
Nervous system	Protection against neuropathy induced by cancer chemotherapeutic drug cisplatin	[158]
Nervous	Response to traumatic brain injury	[159]
Heart	Trastuzumab (Herceptin) cardiotoxicity	[87,88,92]
Vascular	Angiogenesis (in tumor growth)	[160,161]
Breast	Breast tumor formation	[162]
Breast	Paget's disease (α-NRG as motility factor stimulating spread of neoplastic cells)	[163]
Skin	Wound Healing (α-NRG as motility factor for keratinocytes)	[164]
Gut	Hirschsprung's disease	[136]
Limb	Regeneration (newt limb)	[165]

[a] This compilation includes diseases/injuries for which NRG involvement is supported by genetic evidence and/or by manipulation of NRG1 signaling in animal models. The one exception is response to traumatic brain injury and stroke, for which involvement of NRG1 signaling has been proposed solely on the basis of changes in NRG1 expression.

in the pathogenesis of disease, and what are the prospects for disease therapy based on modulating neuregulin signaling? Table 3 summarizes currently investigated pathological and therapeutic considerations with respect to NRG1. Here I will briefly describe only one: the recent evidence for NRG involvement in schizophrenia.

Schizophrenia is a disabling neuropsychological disorder with strong familial characteristics suggestive of a genetic component [62]. A genome wide survey of patients with familial schizophrenia in Iceland, employing both linkage and association methodologies, uncovered the NRG1 gene as a candidate susceptibility gene for schizophrenia, and this association was confirmed in a Scottish population [34,63].

The known activities of NRGs fit well with current hypotheses regarding the neurobiological basis of schizophrenia. One theory proposes that schizophrenia results from a deficiency of glutamatergic innervation relative to dopaminergic innervation. Consistent with the idea that impairment of NRG1 signaling contributes to the pathology of schizophrenia, mice heterozygous for two different mutations in the *NRG1* gene or a null mutation of the *ErbB4* gene display hyperactivity in behavioral tests similar to hyperactivity observed in mice treated with the psychogenic drug phencyclidine (PCP) or with mutations that impair glutamatergic neurotransmission or enhance dopaminergic neurotransmission [34,41,49]. The *NRG1* mutant mice examined in these behavioral studies had an in-frame stop codon introduced within the sequence encoding the NRG1 EGF-like domain [41] (inactivating all NRG1 products of the mutated allele) or introduced within the sequence encoding the transmembrane domain [34]. The phenotype of this latter strain has not yet been reported, but I suspect it will be similar to mice in which the NRG1 cytoplasmic tail has been severely truncated by targeted mutagenesis (see "A tale of the heart . . ." below). Response to the antipsychotic drug clozapine and levels of glutamate receptors were also studied in the mice heterozygous for mutation of the NRG1 transmembrane domain [34]. Treatment with clozapine reversed the hyperactivity of these mice, and they had reduced levels of the NMDA type of glutamate receptors, as assessed by binding of the NMDA receptor ligand MK801. Furthermore, application of soluble NRG1 to cultured neurons stimulates transcription of the NMDA receptor subunit NR2C [64].

Another theory proposes that abnormalities in glial biology contribute to the pathology of schizophrenia [65]. Neuregulins are required for initial differentiation of oligodendrocyte precursors and for their survival [43,48]. A variant of this idea is that a deficiency of glial growth factors—such as NRG—predisposes to synaptic destabilization [66]. It is clear that NRG signaling is required for the stabilization of neuromuscular synapses [67,68], and evidence for NRG involvement in astrocyte biology might implicate NRGs in formation or stabilization of central synapses [69] (see also [70]). A third idea is that schizophrenia results from abnormalities in cortical wiring, and NRGs have been shown to regulate migration of neuronal precursors in culture [71,72]. A fourth hypothesis is that schizophrenia results from abnormalities in synaptic plasticity, and NRG1s inhibit induction of long-term potentiation, a form of synaptic plasticity studied as a model for the neurophysiological substrates of learning and memory [97].

Fig. 1A depicts the boundaries of the genetic haplotype associated with schizophrenia. The only exon of reported NRG isoforms within these bounds is the exon encoding the type II ("GGF2") N-terminal sequence. While widely expressed in the nervous system during development and postnatally [39,73], no in vivo functions of type II NRGs are yet known, and no mutation within this exon that segregates with schizophrenia susceptibility has yet been detected. This raises the possibility that if alterations in NRG signaling are indeed involved in the pathogenesis of schizophre-

nia, the causative mutation may be in intronic or upstream sequence that regulates transcription or splicing.

Sometimes a kiss sent from a distance may be sufficient, but in other situations a kiss on the lips may be required: NRG1 paracrine signaling by shedding and secretion and NRG1 juxtacrine signaling

The ErbB family of receptors and their ligands has been described as a "signaling network" with an input layer comprised of ligands, receptors, and transactivators; a signal processing layer comprised of adapters, cascades, and transcription factors; and an output layer comprised of the biological consequences of ligand-ErbB interaction, such as stimulation of proliferation, inhibition of apoptosis, and differentiation ([1]; see also [98–100] and companion reviews in this issue). In moving from level to level of the network, there is both convergence and divergence, and there are horizontal (lateral) interactions within each level. While we have learned much about the network, we are only beginning to understand how the components of the network are selected, arranged, modified, and modulated in individual cells to achieve physiologically adaptive outcomes of cell-cell interactions.

Much of the recent excitement in the study of cell-cell interactions derives from advances in defining the intracellular signal transduction pathways that couple (translate) receptor activation to cellular responses. However, equally important to understanding cell-cell interactions is defining the mechanisms that regulate the presentation of signals to receptors; that is, understanding the events upstream of receptor activation. Here the role of isoform topology and proteolytic processing in governing NRG-mediated cell-cell interactions will be considered.

Paracrine signaling by Ig-NRGs

Paracrine signaling refers to short distance cell-cell communication mediated by diffusible signaling molecules. Communication mediated by such diffusible signals allows cells not in direct contact to "talk to" each other. Proteins that serve as "paracrine signals" are commonly synthesized as soluble proteins, which—following processing in the ER-Golgi system and transport—are released by secretion, the spilling out of the trafficking vesicle's contents when it fuses with the cell's plasma membrane. Pre-1992, when NRGs were still molecularly unidentified "factors," it was assumed they would turn out to be such typical paracrine signaling proteins, for they were being purified as soluble proteins from medium conditioned by cultured transformed cells and from aqueous (nondetergent) extracts of brain and pituitary and were bioassayed by dissolving the partially purified protein preparations in medium bathing responsive cells [2–7]. Indeed, one NRG isoform—the NRG II-β3 isoform, commonly referred to as GGF2 or simply GGF—is believed to conform to this model. However, most NRGs are synthesized as transmembrane proteins. So, (1) are these transmembrane NRGs released to serve as paracrine signals? And (2) if so, how? The answers are (1) yes, the Type I and (probably) the Type II transmembrane NRGs do generate paracrine signals and (2) the ectodomain is "shed" from the membrane by proteolytic processing [101–107].

The topology and processing of NRGs has been studied principally in cultures of fibroblastic cells in which NRG isoforms have been expressed by transfection [77,82,108–113]. Through such studies, it has been shown that type I TMc-NRGs are expressed as Nout/Cin single-pass transmembrane proteins (type I membrane proteins, not to be confused with a type I NRG) that are cleaved in the "stalk" region to produce a paracrine signal. Type I NRGs (and likely type II NRGs, the other class of Ig-NRGs) appear to act principally as paracrine Type I (short distance, diffusible) signals. Since most type I NRGs in the nervous system are synthesized as transmembrane proteins, paracrine signaling requires proteolytic cleavage of the TMc-NRG proprotein in the stalk region to release the bioactive ectodomain fragment (NTF; see Fig. 2). One example of a cell-cell interaction that appears quite clearly to be mediated by a soluble bioactive NRG1 fragment produced by shedding is the communication between endocardium and myocardium that was discussed below ("A tale of the heart . . . "). Though space precludes more than a passing remark, it must be noted that shedding can produce an autocrine signal (signal-producing cell talks to itself or cells of the same type), as well as a paracrine signal (signal-producing cell talks to cells of a different type). Thus, reported cases of autocrine signaling by NRGs (i.e., [114,115,140,142]) might involve shed TMc-NRGs.

Similar to Ig-NRGs, EGF, TGFα, and most other ligands for the EGFR are synthesized as transmembrane proproteins that are shed to serve as paracrine signals. Knockout of ADAM17 (a.k.a. TACE) has demonstrated that this metalloprotease is essential for the processing of TGFα and likely one or more other ligands of the EGFR [103,116]. ADAM17 and ADAM19 (a.k.a. meltrin-β) have been shown capable of mediating shedding of NRGs from cultured cells [117,118], but their role in governing NRG signal production in vivo remains unknown. Since inhibiting or stimulating metalloproteases is being considered in the therapy of various diseases, including Alzheimer's disease and cancer it will be important to determine the effects of candidate drugs on NRG signaling.

Are CRD-NRGs (type III NRGs) specialized to serve as juxtacrine signals?

Initially it was assumed that like type I NRGs, the type III NRGs with a transmembrane domain C-terminal of the EGF-like domain (TMc-NRGs), such as III-β1a, would be

single pass transmembrane proteins and that stalk cleavage of Type III NRGs would shed a bioactive ectodomain fragment that includes both the "cysteine-rich domain" (CRD) and the EGF-like domain. However, a direct test of this model in which type I and type III NRGs were expressed by transfection in fibroblastic cell lines [77] yielded surprising results: the topology of NRG III-β1a is unlike the topology of NRG Iβ1a, and, in fact, unlike the topology of any previously reported RTK ligand (however, see [119,120] for discussion of an RTK ligand with another interesting topology). The sequences of NRG III-β1a and I-β1a differ only in their N-terminal regions; their sequence from the EGF-like domain through the C-terminus—including the sequence of the TM domain and juxtamembrane segments is identical. However, instead of being an Nout/Cin single-pass transmembrane protein like type I TMc-NRGs, the type III NRGs with a TM-domain C-terminal of the EGF-like domain are Nin/Cin two-pass transmembrane proteins, with a hydrophobic segment within the CRD serving as a second transmembrane domain (see Fig. 2). This has two major consequences: (1) Instead of being an extracellular protein-protein interaction domain as originally suspected, the CRD domain is mostly intramembrane and intracellular. (2) Stalk cleavage of Type III TMc-NRGs does not shed a bioactive ectodomain fragment, but instead creates a transmembrane N-terminal fragment. As would be predicted from the topological differences, when type III and type I NRGs are expressed in parallel cultures, the amount of type III NRG released into the medium is much less than the amount of type I NRG, but the amount of type III NRG exposed at the cell surface—most of which is the transmembrane N-terminal fragment—is much more than the amount of type I NRG [77]. It should be noted also that NRG III-β3, a form lacking the TM-domain C-terminal of the EGF-like domain also accumulates on the cell surface ([79]; J. Wang and D. Falls, unpublished data). These results raise the possibility that type III NRGs are specialized for juxtacrine (direct-contact) signaling, whereas type I NRGs are specialized for paracrine signaling.

There is evidence that type III NRGs do in fact serve as juxtacrine signals in vivo. Schwann cells are the glia of the peripheral nerves. One well-known function of Schwann cells is to myelinate the axons of sensory and motor neurons, thereby dramatically speeding conduction of action potentials. In sensory neuron-Schwann cell cocultures, Schwann cells in contact with sensory neuron axons have a higher proliferation rate than Schwann cells not contacting axons and this growth-promoting activity is blocked by antibodies inhibiting NRG signaling [121,122]. That type III NRGs are an essential component of this contact-dependent signal is suggested by the profound depletion of Schwann cell populations in the type III NRG KO mice ([42]; see Table 2 and discussion above).

A study of mechanisms regulating differentiation of Schwann cells from neural crest progenitors in a cell culture model has both demonstrated juxtacrine signaling by type III NRGs and shown that the consequences of signaling by membrane-bound type III can differ from the effects of signaling by soluble NRG [123]. In these experiments, NRG III-β3 was expressed in a small proportion of the cultured cells using a retroviral vector. As a control, green fluorescent protein (GFP) was expressed using the same vector in parallel cultures. Cells contacting the NRG expressing cells were positive for Schwann cell markers at a significantly higher frequency than cells contacting the GFP-expressing cells, demonstrating the juxtacrine signaling capability of type III NRG. Intriguingly, in similar cultures, soluble (recombinant) type III or type II NRG applied at high concentration was incapable of inducing expression of Schwann cell markers. The proposed proprotein topology and mode of signaling for various isoforms is summarized in Fig. 3. Taken together, the current evidence argues for juxtacrine signaling by type III NRGs and paracrine signaling by types I and II.

Even for a kiss sent from a distance, the tingle can linger: prolongation of Ig-NRG's effect by heparin

The retention of type III NRGs in the membrane of type III expressing cells may not only limit the range of signaling, but also effectively concentrate the signal by confining it to the two-dimensional plane of the membrane. There is recent evidence for an alternative strategy of signal enhancement employed by Ig-NRGs. Each of the protein purification schemes by which NRGs were initially isolated employed a step of heparin chromatography, and each purified an Ig-NRG. In retrospect this is not surprising, as it was subsequently shown the Ig-like domain binds heparin and other highly charged glycosaminoglycans [124]. In contrast, CRD-NRGs do not bind heparin [79].

Glycosaminoglycans are the carbohydrate side chains of proteoglycans, proteins found in the extracellular matrix and on the surface of cells. The affinity of the NRG Ig-like domain for cell-surface and extracellular matrix proteoglycans may provide a mechanism for limiting diffusion of Ig-NRGs and/or creating extracellular reservoirs of NRGs. Ig-NRGs are deposited in the basal lamina of the neuromuscular synapse [125,126]. It has been proposed that Ig-NRGs become bound to the basal lamina by interaction of the Ig-like domain with basal lamina proteoglycans and that the bioactive EGF-like domain is subsequently freed from the matrix by a protease that cleaves the matrix bound N-terminal fragment between the EGF-like domain and the Ig-like domain [124,127].

A new twist to this story is evidence that binding of Ig-NRGs to the surface of cultured muscle through interaction with cell-surface proteoglycans enhances the potency of the Ig-NRG in inducing receptor phosphorylation compared to a recombinant form consisting only of the EGF-like

domain. Furthermore, compared to the EGF-like domain-only form, the Ig-NRG induced a longer period of ErbB receptor phosphorylation and more effectively stimulated synthesis of acetylcholine receptors [128].

A tale of the heart (and of paracrine signaling, Ig-NRGs, the NRG cytoplasmic tail, and NRG trafficking)

As noted above, mice genetically altered so that they produce no bioactive Ig-NRGs (Ig-NRG KOs) have the same cardiac phenotype as the pan-NRG KOs. Mice homozygous for *NRG1* mutation that causes all transmembrane NRG1s (TMc-NRG1s) to have their tail truncated to a length of only three amino acids ($NRG1^{\Delta CT/\Delta CT}$ mice) also have the same cardiac phenotype ([44]; see Table 2). However mice that produce no bioactive CRD-NRGs have not been reported to have cardiac defects. What is the underlying cell biology responsible for these results? Several lines of evidence can be woven together to construct an explanation. (1) The geometry of cardiac development is such that for normal cardiac morphogenesis, the endocardium must signal to cells in the presumptive myocardium with which it is not in direct contact. This requires a paracrine (diffusible) type of signal. As discussed above, the Ig-NRGs (Types I and II) may be specialized for paracrine signaling; whereas, the type III/CRD-NRGs may be poorly released from CRD-NRG producing cells and instead specialized for juxtacrine (direct-contact) signaling [77]. (2) Type I NRGs and low levels of type III NRGs are expressed by the endocardium during embryogenesis, but type II NRGs are not expressed by the embryonic myocardium ([39]; see also [78]). (3) While most released and transmembrane proteins have a classic N-terminal signal sequence that targets the nascent protein to the endoplasmic reticulum, none of the NRGs do. Instead various other sequences in NRG isoforms appear to serve as "cryptic", noncleaved internal signals targeting nascent NRG proteins to the ER-Golgi-export pathway. The β3-NRGs lack the transmembrane domain C-terminal of the EGF-like domain (see Fig. 1). When expressed by transfection in fibroblastic cells, NRG II-β3 is effectively released into the medium [6] and NRG III-β3 is effectively trafficked to the cell surface [79], but NRG I-β3 is not released [2,6]. This suggests that the types II and III N-terminal sequences each contain an ER targeting signal, but that the type I N-terminal sequence does not. Both the type II and III N-terminal regions include a hydrophobic stretch of amino acids, and it is likely that this hydrophobic stretch is all or part of the signal. So does this mean that type I NRGs are a "dead" class of NRG isoforms? Indeed not! Unlike NRG I-β3, the transmembrane type I NRGs (i.e., I-β1a, I-β2a, and I-β4a) are effectively released. Since these type I NRGs with a transmembrane domain carboxyterminal of the EGF-like domain ("TMc-NRGs") are identical with NRG I-β3 from the N-terminus through the EGF-like domain, the TM-NRGs must contain an export pathway targeting sequence downstream of the EGF-like domain. Likely the transmembrane domain serves as a signal-anchor sequence [80,81]. But it turns out that a substantial length of the NRG cytoplasmic tail is also required for trafficking of type I TMc-NRG to the cell surface and their release ([77]; see also [82]). Whether the cytoplasmic tail in conjunction with the TM domain is required for initial targeting type I NRGs to the ER, or—as is the case for TGFα [83–85]—for transport along the export pathway, is unclear.

Now we have sufficient information on the table to synthesize an explanation for the similarity in the cardiac phenotypes of mice with all NRG1s inactivated, only Ig-NRG1s inactivated, and tail-truncated TMc-NRG1s. A paracrine NRG signal is required for endocardial induction of myocardial differentiation, and type III/CRD-NRGs—though expressed at low levels by the myocardium—may not be suitable for paracrine signaling. Type II NRGs may be suitable, but they are not expressed. Type I NRGs are expressed, but type I NRGs without a cytoplasmic tail are not released: i.e., the $NRG1^{\Delta CT/\Delta CT}$ mice would—from today's perspective—be expected to have exactly the same phenotype as a type I NRG null. In summary, what we have learned of NRG's cell biology provides a satisfying explanation of the fact that both the Ig-NRG KO and $NRG1^{\Delta CT/\Delta CT}$ mice have the same cardiac phenotype as the pan-NRG1 KO.

Do NRGs also have functions in the heart beyond early development? The cardiac toxicity of trastuzumab (Herceptin) suggests they do. Trastuzumab is used in the treatment of metastatic breast cancer. It is a humanized monoclonal antibody that binds to the NRG receptor ErbB2 (HER2) which is overexpressed in many breast cancers, and it not only reduces the ligand-independent activation of ErbB2 that occurs in cells highly expressing this protein or expressing mutated ErbB2 by down-regulating ErbB2 [1,86], but also blocks NRG activation of ErbB2/4 and ErbB3/4 heterodimers [1]. A fraction of patients treated with trastuzumab develop dilated cardiomyopathy, reflecting weakening of cardiac muscle contractility [87,88]. Most of the trastuzumab-treated patients that develop cardiomyopathy are also being treated with the chemotherapeutic agent anthracycline. Mouse models with a targeted mutation of ErbB2 affecting only the ventricular muscle [89,90] develop dilated cardiomyopathy closely resembling the pathology in trastuzumab-treated patients. Furthermore, in cell culture neuregulins promote survival and growth of cardiac myocytes, and protect them from anthracycline toxicity [91,92]. Thus, NRGs mediate critical signaling in the adult heart, as well as in the developing heart. In the adult, the endothelium of the cardiac microvasculature may be a source of the (paracrine) NRG signal [91]. Just as NRGs appear to mediate signaling in the adult heart, so NRGs are likely to mediate critical signaling events in the adult nervous system. For example, NRGs and their receptors are widely expressed in the postnatal nervous system [73,93–95], NRG

expression in the brain is upregulated by activity [96], and NRGs can inhibit long-term potentiation (LTP), a model of learning [97]. Thus therapeutic strategies that involve perturbing NRG signaling, such as the use of trastuzumab, must take careful cognizance of the normal functions of NRGs in adult, as well as embryonic, organ systems.

Conclusion

We certainly are just at the beginning of deciphering the functions of NRGs and the mechanisms by which the NRG signaling is shaped and modulated to achieve physiologically adaptive outcomes, but already it is clear the NRGs play critical roles in the functioning of a number of organ systems, both during embryonic development and postnatally. Evidence that aberrations in NRG signaling contribute to the pathology of diseases such as schizophrenia and multiple sclerosis lend additional urgency to expanding our understanding of NRG biology. An appreciation of diversification of signaling through employment of combinations of receptors and variations in intracellular signaling cascades has grown over recent years. Now it seems clear that structural differences in NRG isoforms tailor them for different signaling strategies and requirements, providing considerable additional diversification upstream of receptor activation.

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Note added in proof. A good entry point for access to the wealth of NRG isoform information freely available via the World Wide Web is LocusLink [170,171] (http:www.ncbi.nlm.nih.gov/LocusLink/). To begin accessing the LocusLink neuregulin information, on the LocusLink home page enter “neuregulin” in the query box (without quotation marks), and then click “Go.” This will take you to a page listing neuregulin loci. Note that not all species are included in the LocusLink database. For example, as of December 2002, the frog (*Xenopus*) and chicken (*Gallus*) NRG1 sequences are not referenced in LocusLink. The Mouse Genome Informatics database (MGI) can be accessed from LocusLink or directly (www.informatics.jax.org). Among many other things, this valuable compilation of NRG data includes a listing of each reported targeted mutations of the *NRG1* gene, along with the official nomenclature for each mutation.

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