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Multiple Personalities of Neuregulin Gene Family Members

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In this issue of the *Journal*, Buonanno and his colleagues delineate important differences between neuregulin 1 (Nrg-1) and neuregulin 2 (Nrg-2). Their study provides intriguing new perspectives on neuregulin signaling mechanisms and the role(s?) of Nrgs in making and maintaining synaptic connections. The neuregulin gene family members are located on distinct chromosomes, with the Nrg-1 and Nrg-2 genes giving rise to multiple isoforms as a result of rich differential splicing. Longart et al. demonstrate dissimilar temporal, spatial, and cellular patterns of expression for Nrg-1 and Nrg-2 in vivo. These results provide a framework for evaluating the Nrg-1 and Nrg-2 signaling systems, which appear perfectly poised to mediate distinct, but perhaps complementary, functions in the formation, maintenance, and plasticity of CNS connections.

There are three major results related to Nrg-2 signaling that are set forth in the accompanying paper from Buonanno and colleagues. First, the authors provide additional new insight into the temporal and spatial patterns of Nrg-2 expression, comparing the mRNA profile of Nrg-2 with the profiles of its sibling Nrg-1 and Nrg-3 genes in prenatal, perinatal, and adult mice. Second, the authors delineate the distribution of the Nrg-2 protein within dispersed neurons in vitro: Nrg-2's dendritic localization contrasts with the apparent axonal targeting revealed with Nrg-1 immunoprobe. Third, the authors present evidence that the processing of transmembrane “pro-Nrg-2” proteins differs from that of Nrg-1. The story, while still evolving, is extremely intriguing. These latest findings tantalize us with the notion that the sibling members of this gene family are playing quite different, perhaps complementary, functional roles in establishing CNS synapses.

NEUREGULINS: SO MANY GENES, EVEN MORE ISOFORMS. WHAT DOES IT MEAN?

Four distinct vertebrate genes encode neuregulins, prosaically named *nrg-1*, *nrg-2*, *nrg-3*, and *nrg-4*. Despite the dull nomenclature, this family includes some distinguished and highly distinguishable kinfolk, as reported in the accompanying report from Buonanno and colleagues.

A few introductory notes on the neuregulins and their erbB receptor signaling partners are necessary for the best appreciation of the significance of the Longart et al. findings. In vertebrates, the erbB receptors and their ligands make up a rich and complex signaling network involving receptors encoded by four distinct genes (erbB1–4) and ligands derived from at least 10 different genes (EGF, TGF- α , HB-EGF, amphiregulin, epiregulin, betacellulin, and Nrg-1–4; for review see Yarden and Slivkowski, 2001). Signaling involves ligand-dependent activation of tyrosine kinase activity intrinsic to the receptors. Within the erbB family, erbB1,

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erbB3, and erbB4 bind ligand, whereas erbB2 functions as a general core-ceptor. Once homo- or heterodimers are activated, the intracellular kinase domains autophosphorylate and activate numerous signaling pathways. One persistent question in this field is how signaling specificity is conferred given the range of possible receptor–ligand combinations. The accompanying paper by Longart et al. provides at least two possible ways by which neuregulin-erbB signaling might be distinguished in vivo.

Longart et al., 2004 focuses on a subset of the erbB family ligand network, comparing several aspects of the expression, subcellular targeting, and processing of products of the Nrg-1 and Nrg-2 genes. Although the Nrg-2 gene is somewhat less complex than Nrg-1 (Nrg-2 encodes six to eight isoforms rather than the 15+ reported products of Nrg-1), Nrg-1 and Nrg-2 are closely related genes, sharing the same basic exon/intron organization, giving rise to similar functional domains in the protein products (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Higashiyama et al., 1997; Ring et al., 1999; Yamada et al., 2000; Falls, 2003). The regions with the greatest primary sequence identity between Nrg-1 and Nrg-2 are shown in Figure 1A. Of particular note is that all Nrg-1 and Nrg-2 products include the conserved EGF-like domain (~50% identity). Type I and II isoforms of Nrg-1 and all Nrg-2 isoforms contain Ig-like domains near their N-termini (~35% identity). The EGF-like domain is followed by hypervariable sequences, a transmembrane domain (90% identical), and a relatively long intracellular domain. As a result of alternative splicing in the hyper-variable region that follows the highly conserved EGF-like domain, both Nrg-1 and Nrg-2 products include membrane-anchored and secreted proteins. There are three regions (of unknown function) within the intracellular domains that also share high levels of sequence identity (50–85%).

The human Nrg-2 gene encodes an extended serine-rich N-terminus that has short regions of similarity to the kringle-like domain of the type II Nrg-1 proteins (Ring et al., 1999; Yamada et al., 2000; Fig 1A). Nrg-1 isoforms are divided into three groups based on usage of very distinct first exons (Buonanno and Fischbach, 2001; Falls, 2003). The human Nrg-2 gene is reported to contain a single 5' exon, the second half of which resembles regions of the type II Nrg-1 sequence. Among the original reports of Nrg-2 cDNAs, the DON-1 isoform contained an alternative N-terminus that shares a limited degree of similarity to the type I Nrg-1 N-terminus (Busfield et al., 1997). This raises the possibility that there are still to be identified variants of the Nrg-2 gene.

In view of the structural similarities between Nrg-1 and Nrg-2, it is not surprising that the protein products share the ability to bind to and activate erbB3 and erbB4 receptor tyrosine kinases (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Higashiyama et al., 1997; Crovello et al., 1998; Nakano et al., 2000; Sweeney et al., 2001). Nevertheless, there are differences in the details of the Erb receptor binding profiles of Nrg-1 vs. Nrg-2. The Longart et al. report underscores the potential functional import of such differences. For example, when tumor-derived cell lines are stimulated with recombinant Nrg-1 or Nrg-2, each ligand elicits distinct responses, ranging from the sites of erbB autophosphorylation to the downstream profiles of gene expression induced (Crovello et al., 1998; Sweeney et al., 2001). Despite these intriguing in vitro comparisons of Nrg-2 vs. Nrg-1 signaling via ErbB receptors, relatively little is known of how (or when, or where) Nrg-2 acts in vivo. Longart et al. have begun to remedy this, and, although the results do not yet take us to a full understanding of Nrg-2 functions in vivo, they do provide a well-defined framework for future explorations of the role of different Nrg genes in pre- vs. postsynaptic control of neuronal connectivity.

Nrg-2 DIFFERS FROM Nrg-1 (AND FROM Nrg-3) IN mRNA EXPRESSION PATTERN

By using in situ hybridization probes to unique domains of Nrg-1, -2, and -3, Longart et al. confirm and extend earlier reports that Nrg-1 and Nrg-2 are expressed in distinct and largely nonoverlapping regions of the mouse brain (see, e.g., Carraway et al., 1997). Particularly notable areas of differential expression include the hippocampus, where neurons in CA1 and CA3 express Nrg-1, compared with Nrg-2's predominant expression in the dentate gyrus by postnatal day 7 (P7). Other areas of complementary expression in P7 and adult animals include thalamic, pontine, and lateral amygdaloid nuclei (Nrg-1 positive) vs. olfactory bulb and cerebellum (strongly Nrg-2 positive). In contrast with both of its siblings, Nrg-3 mRNA is expressed throughout the brain, in prenatal to adult animals.

Although these studies emphasize the differences in the spatial patterns of Nrg-1, Nrg-2, and Nrg-3 expression, Longart et al. bring to light the even more striking divergence in the *timing* of expression of these sibling genes during development and in the adult mouse CNS. Nrg-1 appears to be the predominant gene family member expressed in a region-selective manner during the pre- and perinatal periods, after which the mRNA levels fall dramatically. In contrast Nrg-2 is barely detectable in prenatal CNS but displays a strong regional pattern in the postnatal mouse and robust expression in hippocampus, olfactory bulb, and cerebellum that persists into adulthood. How these patterns of mRNA expression might translate into differences in the functional roles of the Nrg siblings is, of course, complex, depending on the relative levels of protein translation, degradation, and targeting, among other factors. Nevertheless, the temporal patterns of expression revealed by these in situ hybridization studies are consistent with the possibility that the early preand perinatal functions of Nrg-1 may be subserved by Nrg-2 in the adult. The generation of postnatal null mutants of Nrg-2, which may be viable in view of the CNS expression pattern, should be extremely informative regarding the role of Nrgs in the making and maintaining of synaptic connections.

DIFFERENTIAL TARGETING OF Nrg-1 VS. Nrg-2 WITHIN THE NEURON

By using newly generated subtype-specific antibodies, Longart et al. compare and contrast the localization of Nrg-1 vs. Nrg-2 protein within individual neurons. In vitro, Nrg-1 distributes to axonal as well as somatic and dendritic compartments, which is consistent with a number of reports of Nrg-1 axonal targeting in vivo (Goodearl et al., 1995; Sandrock et al., 1995; Carroll et al., 1997; Vartanian et al., 1997; Yang et al., 1998; Fernandez et al., 2000). In marked contrast, Nrg-2 is largely excluded from axonal domains. This result raises two questions: First, what processes regulate this differential targeting, and, second, what is the consequence in terms of Nrg-erbB signaling?

Targeting of erbB ligands to distinct subcellular compartments in polarized epithelia has been examined for EGF, TGF α , HB-EGF (Dempsey and Coffey, 1994; Dempsey et al., 1997, 2003; Damstrup et al., 1999; Urena et al., 1999), and more recently Nrg-1 (Vermeer et al., 2003). EGF and TGF α , two alternate ligands for the ErbB1/EGF receptor, accumulate in distinct compartments of polarized kidney cells via apparently distinct mechanisms. TGF α is present on to the basolateral compartment because of directional targeting that requires intracellular domain sequences, including the terminal valine residue (Fernandez-Larrea et al., 1999; Urena et al., 1999; Dempsey et al., 2003). In contrast, accumulation of EGF in apical membranes results from differential processing at the cell surface rather than specific targeting through the secretory pathways (Dempsey et al., 1997). Perhaps differences both in C-terminal sequences and in intracellular domains, which are critical for surface expression of the Nrgs (Liu et al., 1998a,b), as well as differences in processing (see below) contribute to the establishment and maintenance of discrete compartmentalization. Given the preservation of

differential Nrg-1 and Nrg-2 subcellular targeting in dispersed neurons in vitro, these questions should prove amenable to standard molecular and cell biological analyses.

The second question is more difficult to approach but obviously holds the greater significance. Our current understanding of Nrg-1 signaling in the nervous system, supported by both biochemical and genetic studies, involves shipping of Nrg-1 to processes (Goodearl et al., 1995; Sandrock et al., 1995; Carroll et al., 1997; Yang et al., 1998; Fernandez et al., 2000), where it can activate erbB2:erbB3 receptors (predominantly glial; Riethmacher et al., 1997; Steiner et al., 1999; Sherman et al., 2000; Gerecke et al., 2001; Hansen et al., 2001) and erbB2:erbB4 or erbB4 homodimers on neuronal or muscle targets (Ozaki et al., 1997; Rieff et al., 1999; Garcia et al., 2000; Huang et al., 2000; Krainock and Murphy, 2001; Yau et al., 2003). The level of tonic signaling can be controlled by regulating the level of expression of Nrg-1 and/or the rate of shipping and release of Nrg-1 from neuronal processes. The latter process is influenced both by regulated metalloprotease cleavage (in particular for type I Nrg-1 isoforms, also known as *ARIA/NDF*, etc.) (Burgess et al., 1995; Han and Fischbach, 1999a,b; Montero et al., 2000, 2002; Buonanno and Fischbach, 2001; Shirakabe et al., 2001; Falls, 2003) and by the choice of which Nrg-1 isoforms are expressed, resulting in either constitutive release of type II Nrg-1 β 3 isoforms (also known as *GGF*; that lack transmembrane domains; Buonanno and Fischbach, 2001; Falls, 2003) or little or no release in the case of the membrane-tethered type III Nrg-1 isoforms (also known as *CRD-Nrg-1*; Schroering and Carey, 1998; Buonanno and Fischbach, 2001; Wang et al., 2001; Cabedo et al., 2002; Falls, 2003). With targeting of Nrg-2 to dendrites, additional issues arise. First, what is the target for released Nrg-2? Is the target erbB receptors expressed on neighboring cells, or is Nrg-2 acting in a truly autocrine fashion by activating erbB4 receptors that appear to be concentrated in postsynaptic densities by virtue of interactions with PSD95 (Garcia et al., 2000; Huang et al., 2000)?

Other examples of autocrine-type signaling of Nrg-1 and its receptors might be informative. For example, in vitro studies have shown that cells in the epithelial monolayer of lung express both erbB receptors and Nrg-1, yet, under resting conditions, the erbB receptors are not activated (Vermeer et al., 2003). The lack of ongoing ligand–receptor interactions and, hence, constitutive signaling appears to arise from spatial segregation of ligand (Nrg-1 isoform that normally resides on the apical surface of the epithelium), separated by a web of specialized junctional proteins from its receptors (erbB3:erbB2, normally confined to the basolateral surface). Signaling results from disruption of the junctional contacts between adjacent cells that allow “mixing” of the apical and basolateral domains.

It is possible that, in the dendritic arbor, a similar spatial segregation of Nrg-2 and ErbB receptors is maintained. Mixing could result not necessarily from damage as predicted from the strict analogy to the Vermeer et al. (2003) study but from a challenge to basal conditions, such as a strong depolarization, a stimulus sufficient to elicit changes in the organization of pre- and/or postsynaptic domain proteins (Fig. 1B). In this regard, it is worth noting a series of elegant reports from Colman and colleagues. These investigators have proposed that transmembrane adhesion molecules that play a role in segregating membrane domains of epithelia or immune cells might play an analogous role in the organization of pre- and postsynaptic specializations (Benson et al., 2001; Phillips et al., 2001; Dustin and Colman, 2002; Huntley et al., 2002). Depolarization alters the structures of these specializations and the underlying actin cytoskeleton, alterations that influence insertion and distribution of many membrane proteins (for review see, e.g., Huntley et al., 2002). These activity-dependent changes might be the trigger that allows mixing of Nrg-2 and erbB receptors in the postsynaptic domain.

In Figure 1B we present a cartoon that attempts to integrate two of the key findings of Longart et al. with the development of a (highly simplified) neuron–neuron synapse with Nrg/erbB-

expressing components. Early in development, Nrg-1 is shipped to axons, thereby providing a presynaptic signal to target neurons and/or perisynaptic glia via erbB receptor activation (Fig. 1B, blue arrows). By analogy to findings from studies of synapse formation in the periphery, Nrg-1/erbB interactions are predicted to be critical in the setup and initial maintenance of certain CNS synapses. In the postnatal brain, Nrg-1 mRNA expression decreases (perhaps resulting in decreased levels of synaptic Nrg-1 proteins, as illustrated), whereas the levels of Nrg-2 expression (and dendritic Nrg-2) are increased. Thus Nrg-2 becomes the predominant erbB ligand. We propose that interactions between Nrg-2 and postsynaptic erbB4 receptors are minimal as the result of spatial segregation (maintained by transsynaptic macromolecular scaffolding; apropos of Phillips et al. 2001). In this view, synaptic activity remodels the scaffold and allows Nrg-2 access to erbB4, eliciting tyrosine kinase-mediated responses (Fig. 1B, red arrows).

Nrg-1 AND Nrg-2 GENERATE PROTEIN PRODUCTS THAT INTRINSICALLY ARE HANDLED DIFFERENTLY

Another major conclusion from the study of Longart et al. is that Nrg-1 and Nrg-2 are processed in very different manners. This is, perhaps, a subtle point. The data indicate that, in vitro (dramatically) and in vivo (less dramatically), pro-Nrg-2 is less stable than pro-Nrg-1. The authors attribute this to distinct proteolytic pathways acting on each protein. Although this explanation could account for the differences shown, especially when ectopically expressed, there is no direct evidence that the difference in rate of processing results from different enzymes vs. differences in the primary sequence of the substrates. A further point of interest is the apparent continued association of the cleaved extracellular domain with the cell surface. This might reflect, as proposed by the authors, tight interactions with cell-surface-associated heparin sulfate proteoglycans. However, this conclusion is based on parallels with type I/II Nrg-1 isoforms, which both bind tightly to heparin and are efficiently released from the cell surface. The better experimental (if not structural) parallel is with the type III Nrg-1 isoforms that remain membrane tethered as a result of an N-terminal membrane association domain (and possibly an additional lipid anchoring posttranslational modification; Schroering et al., 1998; Buonanno and Fischbach, 2001; Wang et al., 2001; Cabedo et al., 2002; Falls, 2003). Is it possible that the pro-Nrg-2 cleavage is on the cytoplasmic side of the membrane, leaving the extracellular domain still attached to the transmembrane domain? Might other molecular interactions, such as those proposed for type III Nrg-1 β 3 (Cabedo et al., 2002), present processed Nrg-2 in a membrane tethered configuration? Or, as hinted at in the authors' Discussion, is the extracellular domain of Nrg-2 largely vesicular? In this case, the surface pool might be a minor part of the total Nrg-2 whose release could be dependent on signals that stimulate exocytosis. Only experimentation can resolve this issue.

A final point of speculation is that the Nrg-2 intracellular domain may have additional functions. Bao et al. (2003) recently demonstrated a "back-signaling" pathway that is activated concomitantly with translocation of the Nrg-1 intracellular domain to the nucleus. Given the high degree of conservation of the Nrg-1 and Nrg-2 intracellular domains (see Fig. 1A) it is possible that *both* Nrg-1 and Nrg-2 proteins can function as bidirectional signaling molecules.

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Fig. 1.

Comparison of neuregulin-1 and neuregulin-2 structure and function. **A:** Top: Structural organization of Nrg-2 and a type II Nrg-1 isoform. Bottom: An alternate form of Nrg-2 (DON-1) is aligned with type I and type III Nrg-1 β a isoforms. The conserved structural/functional domains are indicated, including the Ig-like domain (not present in type III Nrg-1), the EGF-like domain, and the transmembrane (tm) domain. Note that splice variants of Nrg-1 (e.g., the Nrg-1 β 3 depicted) and Nrg-2 (not shown) result in isoforms that lack the transmembrane domain. Type II Nrg-1 isoforms contain an N-terminal kringle-like domain. The green boxes indicate additional regions of sequence conservation for which no specific functions are known. Although in general the Nrg-2 N-terminus is distinct from Nrg-1 N-termini, there are two regions (a and b) of 30–40 amino acids that share 60% and 40% sequence identity, respectively, between Nrg-2 and the type II Nrg-1 products. In addition, there are three regions in the C-terminal intracellular domain that are conserved (c, d, and e) between Nrg-1 and Nrg-2, sharing about 45% (c), 90% (d), and 85% (e) sequence identity. The Nrg-2 DON-1 cDNA differs from other Nrg-2 cDNAs at the N-terminus (Busfield et al., 1997). In this case, there are only about 30 amino acids before the Ig-like domain that share ~35% identity to the unique type I Nrg-1 N-terminus (designated a'). The origin of this sequence is a bit unclear; reports on the sequence of the human Nrg-2 gene on chromosome 5 do not include an exon corresponding to this region (Ring et al., 1999; Yamada et al., 2000). The approximate locations of the epitopes of the antibodies used to localize Nrg-2 (Ab1394) and Nrg-1 (sc-348) are indicated. **B:** Cartoon of developmental changes and possible signaling mechanisms of Nrg 2. As shown in the paper by Longart et al., Nrg-erbB signaling would initially involve Nrg-1 (blue, shown in axons) activating erbB receptors, either on postsynaptic dendrites (mostly erbB2 + erbB4) or on perisynaptic glia (G; mostly erbB2 + erbB3). These signals would stimulate receptor tyrosine kinase signaling in dendrites and/or glia and, at sites where membrane-tethered Nrg-1 isoforms are expressed, “back-signaling” in the pre-synaptic neuron (blue arrows; Bao et al., 2003). The center panel contrasts the perinatal synapse with an idealized adult synapse. Nrg-2 in the postsynaptic dendrite predominates (as demonstrated in the report of Longart et al). Under basal conditions, signaling via postsynaptic erbB4 might be limited by sequestration of receptors into a subcompartment (perhaps enclosed by macromolecular scaffold of transsynaptic adhesion proteins and the underlying cytoskeleton (represented by the conical structure;)) or by retention of Nrg-2 in vesicles as discussed in the

accompanying paper (or both) (rightmost panel). Activation of the postsynaptic dendrite, as the result of neurotransmitter release from the presynaptic terminal may lead to changes in the macromolecular scaffold and thus to redistribution of Nrg-2 so that it now gains access to the postsynaptic erbB4 receptors.