Characterization of a neural-specific splicing form of the human neuregulin 3 gene involved in oligodendrocyte survival

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Summary

Neuregulins are a family of genes involved in key aspects of neural biology. Neuregulins 1, 2 and 3 (*NRG1*, *NRG2* and *NRG3*) are expressed in the mammalian nervous system. It is well established that *NRG1*, with fifteen different splicing forms, is central for brain development and function. However, the biological relevance of *NRG2* and *NRG3* remains elusive. Here, we report the identification of a new isoform of *NRG3* that is specifically expressed in the human embryonic central nervous system. Sequence alignment with the human genome suggests that this transcript is produced by alternative promoter usage. The encoded polypeptide is a type-I-glycosylated plasma membrane protein, which is shed into the extracellular space where it activates erbB4, a pivotal receptor for brain development. In addition, we show that the protein has a signal sequence that is cleaved after membrane insertion. Proteasome inhibition with Lactacystin enhances the expression of the protein, whereas impairment of ubiquitylation in the conditional mutant cell line ts20 protects the protein from degradation. These observations imply that the ubiquitin/proteasome pathway regulates biogenesis of the protein. We also show that recombinant neuregulin 3 acts as an oligodendrocyte survival factor by activating the phosphoinositide 3-kinase signalling pathway. Therefore, we report a new post-translationally regulated isoform of neuregulin 3 expressed in the developing human central nervous system with a role in oligodendrocyte survival.

Key words: EGF-like, Alternative splicing, Ubiquitin/proteasome system, ErbB4 receptor, Oligodendrocyte apoptosis

Introduction

Neuregulin 1 (*NRG1*) gene products form a family of neurally expressed proteins that perform a wide range of functions in the developing nervous system, such as controlling the number of acetylcholine receptors at the neuromuscular junction, promoting Schwann cell proliferation and survival, inducing differentiation of oligodendrocytes and regulating the tangential migration of cortical interneurons (Falls, 2003; Flames et al., 2004). *NRG1* has been identified recently as a potential susceptibility gene for schizophrenia (Corfas et al., 2004). *NRG1* gene products are also expressed in non-neural tissues, where they are involved in heart and breast development (Falls, 2003; Zhao et al., 1999). The *NRG1* gene subfamily is broad, as evidenced by the 15 isoforms described so far. This molecular diversity is further increased by the presence of three additional genes encoding for neuregulins in mammals, namely *NRG2*, *NRG3* and *NRG4* (Falls, 2003). Whereas *NRG4* has not been found to be expressed in neural tissues, *NRG2* and *NRG3* are abundantly expressed in the mammalian central nervous system (CNS) (Falls, 2003; Longart et al., 2004). *NRG3* mRNA is expressed in the mouse brain from embryonic day (E) 13 to adult (Zhang et al., 1997; Longart et al., 2004). *NRG3* is also expressed in non-neural tissues, where it is involved in mammary gland development and carcinogenesis (Dunn et al., 2004; Howard et al., 2005).

A previously described isoform encoded by the *NRG3* gene contains a characteristic, extracellular epidermal growth factor (EGF)-like domain which, when purified from genetically engineered bacteria, can bind and activate erbB4, but not erbB2 or erbB3 receptors (Zhang et al., 1997), suggesting a non-overlapping role with *NRG1*. However, no physiological or cellular expression studies have been performed on *NRG3* gene products yet.

ErbB4 homozygous knock-out mice show aberrant migration of a subpopulation of hindbrain-derived cranial neural crest cells, which – along with heart trabeculae defects – cause death during mid-embryogenesis (Tidcombe et al., 2003). Furthermore, the erbB4 pathway has been proposed to participate in several aspects of neural cell biology, such as cortical interneuron migration (Flames et al., 2004), synaptic plasticity (Huang et al., 2000), neuronal cell death (Goldshmit et al., 2001), long-term potentiation (Eilam et el., 1998; Huang et al., 2000; Ma et al., 2003) and oligodendrocyte biology (Calaora et al., 2001; Schmucker et al., 2003; Sussman et al., 2005). All these findings correlate with the nervous-system-specific expression pattern of *NRG3* and suggest that this gene could be involved in brain development and/or function.

NRG1 is a highly spliced gene with at least 15 splicing forms described so far (Falls, 2003). Alternative splicing in genomes is a major mechanism for generating proteome diversity

(Nurtdinov et al., 2003). Recent analysis has estimated that 60% of human and 41% of mouse genes are alternatively spliced. Like the *NRG1* subfamily, it seems likely that the *NRG3* gene suffers post-transcriptional processing, giving rise to a diverse family of splicing forms. Here, we have addressed this issue and used a computer-based search of expressed sequence tag (EST) databases to identify new isoforms of the *NRG3* gene. We report the recognition of a novel *NRG3* splicing variant – human foetal brain neuregulin 3 (hFBNRG3) – isolated from a human foetal brain cDNA library. Using reverse transcriptase (RT)-PCR we show that this transcript is specifically expressed in the human foetal CNS, but not in other tissues. The hFBNRG3 product is a 56 kDa protein that differs at the N-terminus from human neuregulin 3 (hNRG3), a previously described isoform of *NRG3* (Zhang et al., 1997). The protein is glycosylated and its lifetime is strongly regulated by the ubiquitin/proteasome pathway. EGF-like activity from hFBNRG3 can be found in conditioned media of transiently transfected mammalian cells, suggesting that the protein is shed into the extracellular space. Notably, when the recombinant protein is added to oligodendrocyte cultures, it promotes oligodendrocyte survival by activating the phosphoinositide 3 (PI 3)-kinase pathway. Taken together, our results are consistent with a model in which a highly regulated *NRG3* gene isoform is expressed in the developing CNS, where it is involved in oligodendrocyte survival.

Results

A novel isoform of the *NRG3* gene is expressed in a human foetal brain library

We used a computer-based approach to search EST databases for sequences identical to the EGF-like motif of *NRG3*. The inquiry identified a full-length cDNA corresponding to a putative new isoform of the *NRG3* gene. The clone was named hFBNRG3 (human foetal brain NRG3) because it was found in a human foetal brain library (clone CS0DF004YG18, GenBank accession numbers AL534571 and AL565572) at the full-length cDNA collection of RESGEN (Invitrogen Corporation). After nucleotide sequencing of the insert and BLAST-searching the human genome, we found that this transcript is an alternative splice product of the *NRG3* gene. Unlike hNRG3, a previously described splicing form, exon 1 is spliced out of the hFBNRG3 transcript and two additional
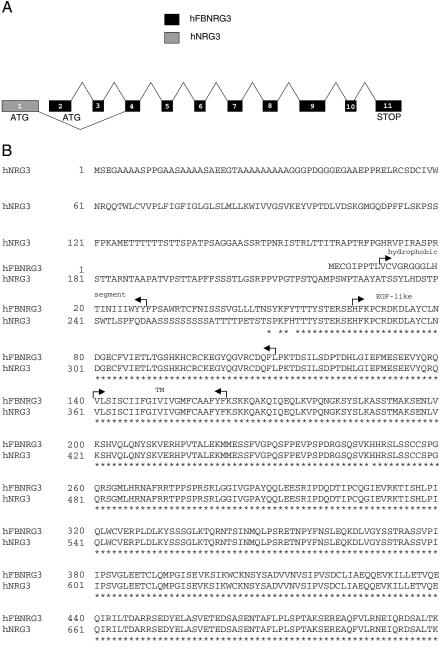

Fig. 1. Sequence comparison of hFBNRG3 and hNRG3. (A) Alignment of the hFBNRG3 and hNRG3 transcripts to the human genome by BLAST search. Exon 1 is spliced out in hFBNRG3, whereas exons 2 and 3 are spliced out in the hNRG3 transcript. Exons 4 to 11 are shared by both transcripts. (B) Predicted aa sequences for both transcripts are compared. EGF-like domains and transmembrane segments are indicated, and also the N-terminal hydrophobic segment for hFBNRG3. Both polypeptides diverge completely in the N-terminal domain and are preserved from the EGF-like to C-end. Perfectly conserved aa are underlined with asterisks.

exons (2 and 3) are included (Fig. 1A). The absence of exon 1 in the hFBNRG3 transcript suggests that a different promoter is used to produce this mRNA. The isolated clone contained an open reading frame (ORF) encoding a protein with a predicted molecular mass of 56 kDa. The encoded polypeptide is identical to hNRG3 from the EGF-like domain to the C-terminus, but differs greatly in both size [54 versus 275 amino

acids (aa)] and sequence (8.4% aa identity) at the N-terminal domain (Fig. 1B). Hydropathy analysis indicates the presence of two hydrophobic segments that are located in the N-terminal region (aa V10-F29 and V140-F162). Segment V140-F162 is highly hydrophobic and long enough to span the width of the plasma membrane. Thus, it probably is the unique membrane-anchor domain of the protein, akin to some isoforms of the *NRG1* gene (Cabedo et al., 2002). The predicted extracellular segment of the protein contains several putative O- and N-glycosylation sites. However, in contrast to most of the *NRG1* splicing forms, hFBNRG3 does not contain IgG-like or kringle domains, a property that is shared with the previously reported hNRG3 isoform (Zhang et al., 1997). The large C-terminal domain (Y163-K499) is probably cytoplasmic, and accommodates a coiled-coil region (L433-G460) and a dileucine motif (L433-L434), suggesting that it is involved in DNA binding, protein-protein interactions or protein sorting and trafficking.

hFBNRG3 is specifically expressed in the human embryonic CNS

To study the expression pattern of the hFBNRG3 mRNA, RT-PCR from several human embryonic tissues was performed. Using specific primers to amplify the complete ORF of hFBNRG3 mRNA we amplified the cDNA from human foetal brain, kidney, testis, spleen, muscle and liver supplied in the Multiple-Choice First-strand cDNA (OriGene Technologies, see Materials and Methods). As shown in Fig. 2, a band of the expected molecular size was amplified from the human foetal brain cDNA, but not from other tissues. The housekeeping β-actin mRNA was used as control (Fig. 2, bottom). To verify the identity of the amplified band, the PCR product was cloned
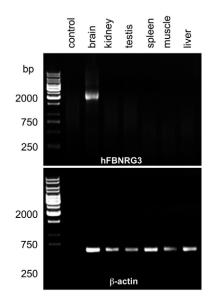
using TA technology in the pCRII dual-promoter vector and sequenced using T7 and SP6 primers; their sequences corresponded to the 5′- and 3′-end of the mRNA encoding the hFBNRG3 protein, respectively. These results, therefore, imply that the hFBNRG3 transcript is expressed in human foetal brain.

Subcellular localization of hFBNRG3 protein in mammalian transfected cells

We next investigated the expression of hFBNRG3 protein in cultured mammalian cells. The ORF of hFBNRG3 was cloned in a mammalian expression vector with a myc-epitope at the C-terminus of the protein. COS-7 cells were transfected with hFBNRG3-myc and the expression of the protein was studied by immunocytochemistry. As shown in Fig. 3A, expression of hFBNRG3-myc protein could be detected in transfected cells and was increased by proteasome inhibition with MG-132 (see below). By contrast, an inversely oriented (inv) hFBNRG3
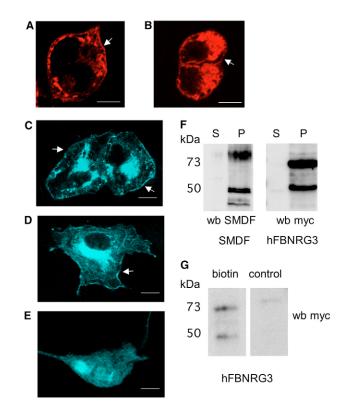

Fig. 3. hFBNRG3 is a surface-exposed plasma membrane protein. (A) Confocal microscopy images show plasma membrane location of the hFBNRG3 polypeptide (COS-7 cells were transfected with hFBNRG3-myc, incubated with MG-132 and processed for immunocytochemistry as described in Materials and Methods). (B) hFBNRG3 is also inserted to the plasma membrane of the neuronal-model cell line PC12. (C) CFP-hFBNRG3 polypeptide localizes on the plasma membrane of COS-7 cells. (D) CFP-SMDF, a plasma membrane neuregulin, was used as control. (E) The soluble CFP showed a wide distribution pattern. Arrows indicate plasma membranes. Bars, 10 μm. (F) hFBNRG3 is considerably associated with the insoluble fraction of crude plasma membrane preparations (P) but not with the soluble fraction (S). SMDF was used as a control. (G) Biotinylation increased the recovery of neuregulin in the streptavidin precipitates, showing that hFBNRG3 is a surface exposed polypeptide. Lysate of nonbiotinylated cells was used as control.

Fig. 2. hFBNRG3 is expressed in the human foetal brain. RT-PCR was used to detect mRNA expression in the human foetal tissues. An amplicon of the expected size was obtained from human foetal brain but not from other foetal tissues (top). The molecular identity of the amplicon was verified after its cloning and sequencing. Quality of cDNA synthesis was checked by amplifying the housekeeping gene β-actin (bottom).

construct [(inv)-hFBNRG3] did not express any protein (data not shown). The polypeptide segment encompassing V140-F162 of hFBNRG3 is highly hydrophobic and long enough to span the width of the plasma membrane, thus it probably is a membrane-anchor domain for the protein. To check this possibility, we used different approaches. First, using the anti-myc antibody, we performed immunocytochemical studies on hFBNRG3-transfected COS-7 cells. Confocal images suggested a membrane localization of the protein (Fig. 3A). This notion was supported by confocal microscopy of cells transfected with construct (CFP)-hFBNRG3 [cyan fluorescent protein (CFP) coupled to hFBNRG3]. CFP-hFBNRG3 displayed a cortical distribution of the fluorescence (Fig. 3C) similar to CFP-SMDF (Fig. 3D), a well documented plasma membrane protein (Cabedo et al., 2002). By contrast, the cytoplasmic protein CFP showed a homogenous distribution pattern (Fig. 3E). Neuregulins are expressed in neural tissues. To gain insight of the subcellular location of hFBNRG3 in a neuron-like cell, immunocytochemistry was performed in transfected phaeochomacytoma 12 (PC12) cells. hFBNRG3 was detected in the plasma membrane of PC12 cells (Fig. 3B) suggesting that, in neuron-like cells too, the protein is located at the plasma membrane.
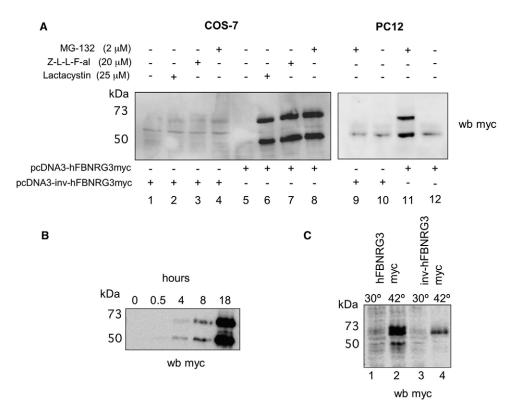
These findings were substantiated further by biochemical methods. Enriched plasma membrane fractions from hFBNRG3-expressing COS-7 cells were western blotted with anti-myc antibody. As shown in Fig. 3F, most of the immunoreactivity was found in the pellets, not in the soluble fraction, as is the case for the membrane protein SMDF. These results demonstrate a plasma membrane location of the hFBNRG3 polypeptide.

Finally, to know whether the protein is exposed on the cell surface, we performed biotinylation experiments on hFBNRG3-expressing cells. When biotin is added to cells in culture it binds only to proteins exposed to the cell surface. As depicted in Fig. 3G, anti-myc immunoreactivity was clearly found in the streptavidin pellets of the biotinylated cells, supporting the notion of a cell-surface-exposed protein. Taken together, our results demonstrate that hFBNRG3 is a membrane protein that is expressed and accessible on the cell surface.

The ubiquitin/proteasome system controls hFBNRG3 biogenesis

During our experiments we noticed that the proteasome inhibitor MG-132 increased the number of hFBNRG3 immunoreactive cells. Therefore, we asked whether the cellular stability of this polypeptide is regulated by proteasomal degradation. Transiently transfected COS-7 cells were incubated with the proteasome inhibitors MG-132 or Z-Leu-Leu-Phe-CHO or the highly specific inhibitor Lactacystin for 18 hours. Thereafter, cells were lysed, and cell extracts subjected to SDS-PAGE, transferred to nitrocellulose membranes and blotted with polyclonal anti-myc antibody. Under our conditions, the hFBNRG3-myc polypeptide could not be immunodetected in western blots unless proteasomes were inhibited. Incubation of the cells with the proteasome inhibitors promoted the expression of two immunoreactive bands of approximately 50 and 70 kDa (Fig.
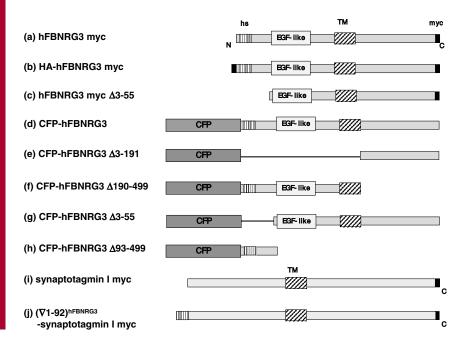

Fig. 4. The ubiquitin/proteasome system regulates the protein stability of hFBNRG3. (A) Proteasome inhibition allows the detection of hFBNRG3 protein expression in anti-myc western blots of transiently transfected COS-7 cells. Nontreated cells (lane 5) showed no immunoreactivity at these conditions. Treatment with three different proteasome inhibitors Lactacystin, Z-Leu-Leu-Phe-CHO (Z-L-L-F-al) and MG-132 (lanes 6, 7 and 8, respectively) resulted in the appearance of immunoreactivity. The transfection of the inversely oriented cDNA produced no immunoreactivity (lane 1), even in the presence of the proteasome inhibitors (lanes 2, 3 and 4). Similar results were obtained in the neuron-like PC12 cells (lanes 9 to 12). Although a faint band was detected in nontreated PC 12 cells (lane 12) it was shown to be unspecific because it was also present in cells transfected with inversely oriented cDNA. (B) Time course of protein stabilization after proteasome inhibition with 2 μM MG-132 in COS-7 cells. As shown, immunoreactivity was already detected after 4 hours of proteasome inhibition. (C) Impairment of the ubiquitylation machinery by incubating the cells at 42°C increased the protein stability in the mammalian conditional mutant cell line ts20. An unspecific band of ~65 kDa was detected at 42°C even in cells that had been transfected with inversely oriented cDNA.

4A). Notice that, the fast-migrating band displays a slightly lower molecular mass than that predicted from the sequence, suggesting postranslational processing of the protein. Likewise, the 70 kDa immunoreactive band suggests that the polypeptide is post-translationally modified. As expected, hFBNRG3-type bands were not observed in cell extracts from cells transfected with the inversely oriented cDNA (Fig. 4A). Similar results were obtained with the neuron-like cell line PC12 (Fig. 4A), substantiating the notion that the protein stability is cell type independent. To gain further insights into protein expression, we performed a time-course study of protein stability after proteasome inhibition in COS-7 cells. As displayed in Fig. 4B, hFBNRG3 can be detected after 4 hours of proteasome inhibition. Similar results were obtained with Lactacystin (data not shown). Taken together, our results suggest that hFBNRG3 is degraded, at least in part, by the proteasome, although the participation of other cellular proteases or lysosomes can not be completely ruled out.

The proteasome is known to degrade ubiquitylated proteins. Ubiquitin is an 8 kDa peptide that is attached to the amide group of lysines in polypeptide chains that are destined for degradation. Multi-ubiquitylated chains of at least four ubiquitin molecules are necessary for efficient recognition and degradation of ubiquitylated proteins by the proteasome (Pickart, 2004). Hence, we next investigated the role of this post-translational modification in hFBNRG3 stability. For these studies, we used the cell line ts20, that harbours a mutant E1 (ubiquitin ligase), active only at the permissive temperature of 30°C, and inactive at the nonpermissive temperature of 42°C (Kulka et al., 1988). To investigate the role of ubiquitylation in hFBNRG3 biogenesis, we transfected hFBNRG3-myc and (inv)-hFBNRG3-myc into ts20 cells, and monitored the expression of the protein at permissive and nonpermissive temperatures. As illustrated in Fig. 4C, anti-myc immunoreactivity was not found in cell extracts when the cells were incubated at the permissive temperature for 18 hours. By contrast, the nonpermissive temperature induces the appearance of the 50 and 70 kDa immunoreactive bands.

Notice that, the ~65 kDa band is unspecific, given that it was also found in the (inv)-hFBNRG3-myc transfected cells incubated at 42°C. These results suggest that hFBNRG3 can be degraded by the proteasome.

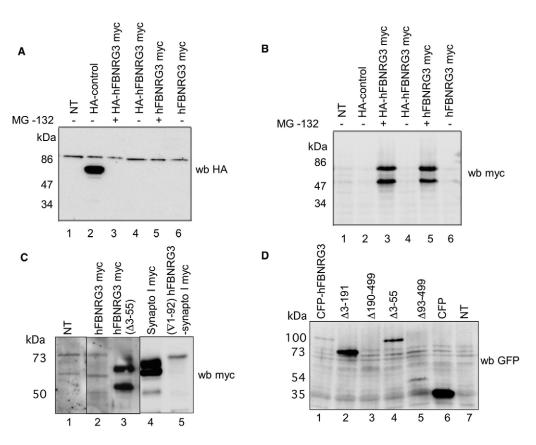### hFNRG3 has a cleavable, destabilizing signal sequence at its N-terminus

Unexpectedly, the hFBNRG3 protein appears on immunoblots as two different forms of approximately 50 and 70 kDa. The fast-migrating band is consistent with proteolytic post-translational processing of the protein, presumably by truncation of a signal peptide. To reveal the molecular identity of the polypeptide with the approximate size of 50 kDa, an HA epitope was fused to the N-terminal domain of the hFBNRG3-myc construct and used as a reporter. Unlike an HA-tagged control protein, expression of the HA-hFBNRG3-myc protein was not detected on western immunoblots from cell extracts (Fig. 6A). The lack of HA-hFBNRG3-myc expression was not due to a defect in protein biogenesis because immunoblotting with an anti-myc antibody showed that the HA-tagged neuregulin was expressed at a similar level to hFBNRG3-myc (Fig. 6B). Notice that the expression of both proteins was only detected when the proteasome was inhibited. These findings indicate that the N-terminus of the protein is proteolytically processed, and suggest that the hydrophobic segment V10-F29 is a signal-sequence that is cleaved and degraded during the insertion of the protein into the plasma membrane.

To determine the molecular determinants of protein instability, we deleted the first 52 aa in the N-terminus of hFBNRG3 [hFBNRG3-myc(Δ3-55)] (Fig. 5). In contrast to hFBNRG3-myc, the mutated neuregulin appeared in western immunoblots as two distinct bands of approximately 50 and 65 kDa even in the absence of proteasome inhibitors (Fig. 6C), indicating that the protein lacking the N-terminus is highly stable in cells. Similar results were obtained with deletion mutants of the CFP-hFBNRG3 protein. As illustrated in Fig. 6D, deletion mutants that do not have the N-terminal domain were readily detected. These results suggest that the N-terminus of the protein conveys instability to the protein. Indeed, fusion of the N-terminal domain of hFBNRG3 to the type I membrane protein synaptotagmin I [(∇1-92)^{hFBNRG3}-synaptotagmin I-myc], significantly decreased the stability of synaptotagmin (Fig. 6C). Taken together, our data indicate that

Fig. 5. Constructs used in this study. The ORF from hFBNRG3 was cloned in-frame with a myc epitope at the C-terminus of the protein (a) or an HA epitope in the N-terminal domain (b). Amino acids 3 to 55 were deleted in the myc-tagged construct (c). The ORF of hFBNRG3 was cloned in-frame after CFP (d). Extracellular and transmembrane (e) intracellular (f) or N-terminal domains (g) were deleted from the CFP-hFBNRG3 construct. A construct bearing the N-terminal domain and part of the EGF-like domain attached to CFP was also produced (h). Synaptotagmin I with a myc-epitope at the C-terminus (i) and a chimeric protein containing the 1-92 N-terminal aa of hFBNRG3 fused to synaptotagmin I myc (j) were also used.

Fig. 6. The N-terminal domain bears a cleavable signal sequence and confers instability to hFBNRG3. (A) Attachment of the HA epitope to the N-terminal domain of hFBNRG3-myc produced no immunoreactivity in anti-HA immunoblots (lane 4) even in the proteasome inhibited cells (lane 3), whereas an HA-tagged control protein was clearly detected (lane 2). As expected, HA immunoreactivity was not detected in non-HA-tagged hFBNRG3 (lanes 5 and 6) and nontransfected cells (lane 1). The band of ~90 kDa present in the immunoblots is unspecific as demonstrated by its presence in the nontransfected cells. (B) HA-hFBNRG3-myc was expressed at levels comparable with those of hFBNRG3-myc as demonstrated by the anti-myc immunoblot of aliquots of the same cell extracts. (C) Protein stability is increased when the peptide harbouring the signal sequence is deleted (lane 3). The attachment of the peptide to the transmembrane protein synaptotagmin I downregulates its expression levels in transfected cells (lane 5). (D) N-terminal domain bearing CFP chimeras (lanes 1, 3 and 5) were hardly detectable in anti-GFP immunoblots, whereas peptide-deleted chimeras (lanes 2 and 4) showed a considerable increase in protein levels. COS-7 cells were transfected with the indicated vectors and cell extracts were immunoblotted with anti-myc, anti-HA or anti-GFP antibody.

hFBNRG3 bears a cleavable signal-sequence and a downregulation signal at its N-terminal domain.

hFBNRG3 is glycosylated and shed into the extracellular medium

We next investigated the molecular identity of the 70 kDa polypeptide detected on western blots. Because the protein contains several glycosylation sites, we first focused on the question whether the protein is glycosylated in cells and, if so, what type of sugar is attached. Post-translational modification with sugar derivatives has been shown to affect the aggregation state and subcellular localization of other neuregulins (Cabedo et al., 2004). To address this issue, we took advantage of the sugar-specificity of two agarose-conjugated lectins. The *Triticum vulgaris* lectin wheat germ agglutinin (WGA) binds to β-GlcNac-modified proteins, whereas soybean agglutinin (SBA) from *Glycine max* interacts with both, α- and β-Gal-Nac-glycosylated proteins. Whole-cell extracts from hFBNRG3-myc-transfected COS-7 cells were incubated with WGA- or SBA-agarose and the presence of the protein in the precipitates was evaluated by western blots using the anti-myc antibody. As depicted in Fig. 7A, a 70 kDa polypeptide was precipitated by the SBA resin but not by WGA. Notice that galactose eluted the slow-migrating band (lane 6), ruling out the possibility of nonspecific binding to the resin. Thus, this result demonstrates that the 70 kDa polypeptide is an α- or β-Gal-Nac-glycosylated form of hFBNRG3.

The EGF-like domain of neuregulins is released into the extracellular medium to activate erbB receptors expressed in target cells. To test this, conditioned medium from cells transiently transfected with hFBNRG3 was concentrated and assayed for erbB4 autophosphorylation in erbB4-transfected COS-7 cells. To validate our assay, a dose-response curve for the activation of the erbB4 receptor was performed with increasing amounts of recombinant glutathione-S-transferase (GST)-NRG3 purified from bacteria (Fig. 7B). Quantification of the blot showed that detection of as low as 3 nM of the recombinant protein was possible. As illustrated in Fig. 7C, conditioned medium from MG-132-treated, hFBNRG3-expressing cells (lane 6) induced a noticeable increase in erbB4 tyrosine phosphorylation. The increase in erbB4 activation was not due to higher load of protein, as demonstrated by reprobing the same blot with an anti-PKCα antibody (bottom). Conditioned medium from hFBNRG3-transfected cells that had not been treated with MG-132 did not induce statistically significant erbB4 phosphorylation (lane 4). Therefore, our results suggest that, hFBNRG3 can be released into the extracellular medium where it can activate erbB4 receptors.

NRG3 is a survival factor for oligodendrocyte precursors

Neuregulin 3 is expressed during mammalian CNS development (Longart et al., 2004). It has been shown that erbB3 activation by neuregulin 1 has an anti-apoptotic effect on Schwann cells (Dong et al., 1995). Recombinant neuregulin
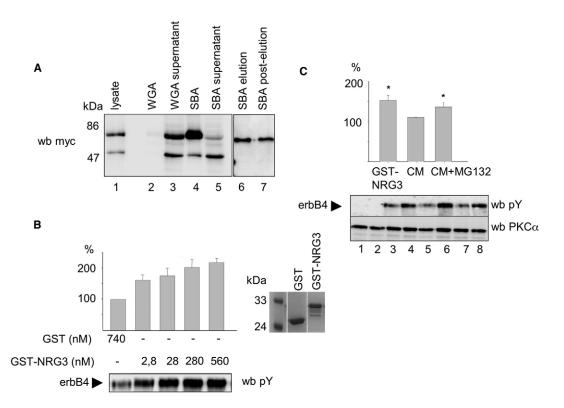
Fig. 7. hFBNRG3 is exported to the plasma membrane and released to the extracellular medium. (A) hFBNRG3 is post-translationally modified with N-acetyl-galactosamine but not with N-acetyl-glucosamine as demonstrated by the absence of affinity for the WGA lectin (lanes 2 and 3) and its binding to SBA lectin (lanes 4 and 5). Nonspecific binding was ruled out by elution with galactose (lane 6 and 7). (B) Dose-response curve of erbB4 phosphorylation. COS-7 cells transiently transfected with pcDNA3-erbB4 were trypsinized and re-seeded to assure identical levels of erbB4 receptor expression per well; after serum deprivation, cells were incubated with the indicated amount of recombinant NRG3 (inset) for 5 minutes, harvested with sample buffer and western blotted with anti-phosphotyrosine antibody. Increases in phosphotyrosine levels were normalized after re-probing the membranes with anti-PKC-α. The results of three different experiments are given as the mean + s.e.m. (top). (C) The same approach was used to detect neuregulin activity in conditioned medium (CM) of hFBNRG3 transfected cells. As shown, erbB4 phosphorylating activity was found in the medium of MG-132-treated, transiently transfected cells (lane 6). Although in some experiments neuregulin activity in the medium of nontreated cells was detected, differences were not statistically significant (lane 4 and bar labelled CM). Basal phosphorylation of erbB4 was estimated from conditioned medium of cells transfected with the inversely oriented hFBNRG3 cDNA without (lane 3) or with (lane 5) MG-132 and also in GST-treated cells (lane 7), and subtracted from calculations. Positive phosphorylation control was performed by adding recombinant GST-NRG3-EGF-like protein (lane 8). No protein bands were detected in nontransfected COS-7 cells even when GST or recombinant neuregulin was added (lanes 1 or 2, respectively). Data are given as mean $\pm$ s.e.m (n=3, *$P \leqslant 0.05$); Student's t-test was used.

1 has also been shown to act as a survival factor for oligodendrocyte precursors (Flores et al., 2000). Oligodendrocytes express erbB2, erbB3 and erbB4 receptors (Schmucker et al., 2003; Park et al., 2001). Since erbB3 is dispensable for oligodendrocyte development both in vitro and in vivo (Schmucker et al., 2003), and erbB2 does not bind neuregulin ligands (Burden and Yarden, 1997), we reasoned that *NRG3* gene products – through erbB4 signalling – might also be involved in oligodendrocyte survival. To address this issue, we studied the effect of recombinant NRG3 on the survival of serum-deprived oligodendrocyte-precursor-enriched primary cultures (Lubetzki et al., 1991). After 7 days in vitro, cultures were serum-starved and GST or GST-NRG3 was added at 150 nM. Forty-eight hours later, cells were immunostained with the anti-NG2 antibody as an oligodendrocyte-precursor-marker and counter-stained with Hoechst dye (Fig. 8A). Apoptotic nuclei of NG2-positive cells were counted for both treatments. Images of five microscopic fields per slide from five different slides per treatment (~750-

1500 NG2-positive cells) were evaluated in each experiment. The results of three independent experiments are shown in Fig. 8B. Serum-starved cultures showed 8.8±0.5% of apoptotic oligodendrocyte precursors. Recombinant neuregulin 3 decreased the number of apoptotic oligodendrocytes to 4.5±0.9%, close to the level of apoptosis in nonstarved cultures (3.5±0.7%).

Akt is a protein kinase that promotes cell survival by inhibiting apoptosis through its ability to inactivate caspase 9 and the transcription factor Bad (Franke et al., 1997). It has been shown previously that neuregulin signalling can activate the PI 3-kinase pathway (Flores et al., 2000) leading to Akt activation by phosphorylation of Ser473. To test whether NRG3 activates the PI 3-kinase pathway in glial cells we studied the effect of GST-NRG3 on Akt phosphorylation in serum-deprived C6 glioma cells. As shown in Fig. 8C, GST-NRG3 promoted the phosphorylation of Akt in a dose-dependent way. The time course of Akt phosphorylation in NRG3-treated serum-deprived

oligodendrocyte-precursor cultures was investigated to determine whether the anti-apoptotic effect of NRG3 in oligodendrocyte precursors can also be mediated by Akt activation. Notably, GST-NRG3 induced the phosphorylation of Akt after 5 minutes of incubation (Fig. 8D). These results suggest that NRG3 acts as a survival factor for oligodendrocyte precursors by activating the PI 3-kinase pathway.

To test this hypothesis further, we checked whether inhibition of PI 3-kinase blocks the cell-survival-inducing activity of NRG3. Oligodendrocyte-precursor cultures were serum-starved and treated with 150 nM of GST or GST-NRG3, with or without 20 μM of the PI 3-kinase inhibitor LY294002. The result of three independent experiments is depicted in Fig. 8E. In this series of experiments GST-treated serum-starved cultures contained 13.7±1.5% of apoptotic oligodendrocyte precursors; LY 294002 did not induce a significant change in the number of apoptotic cells (13.7±3.3%). However, LY294002 reduced the number of NG2-positive cells in our cultures suggesting that, as in other cell systems (Jeong et al., 2005), PI 3-kinase inhibition can induce cell-cycle arrest in oligodendrocyte precursors. Recombinant NRG3 decreased the number of apoptotic oligodendrocytes to 6±0.6% and this effect was completely reversed by co-incubation with LY 294002 (17.7±2%). Taken together, our results show that the anti-apoptotic effect of NRG3 is mediated by activation of the PI 3-kinase pathway.

Discussion

NRG1 gene products regulate numerous aspects of neuronal biology, such as interneuron migration, and fate, differentiation and proliferation of glial cells, as well as synaptic plasticity and the expression of diverse neurotransmitter receptors (Falls, 2003; Rieff et al., 1999). *NRG2* and *NRG3* mRNAs have also been found to be expressed in the mammalian CNS (Longart et al., 2004). However, so far, their biological role is undetermined. Expression of the *NRG3* gene is restricted to the CNS (Zhang et al., 1997), suggesting that this gene has a key role in neural biology. Here, we used a computer-based approach to identify new *NRG3* transcripts. We report a new transcript of *NRG3* (denoted hFBNRG3), whose expression is restricted to the human embryonic brain.

Alignment with the human genome suggests that this transcript is produced by alternative splicing and different promoter usage. Alternative splicing (observed in approximately 50% of mammalian genes) is a crucial step in the regulation of many vertebrate gene products and is thought to have a pivotal role in the generation of proteomic diversity (Nurtdinov et al., 2003; Pan et al., 2004; Lee et al., 2003). Three main types of alternative-splicing cassettes have been described. Conserved alternative splicing is the less common process in mammalian genomes (approximately 15% of the spliced genes). Species-specific alternative splicing of conserved exons, by contrast, is much more frequent and has been estimated to affect approximately 35% of the spliced
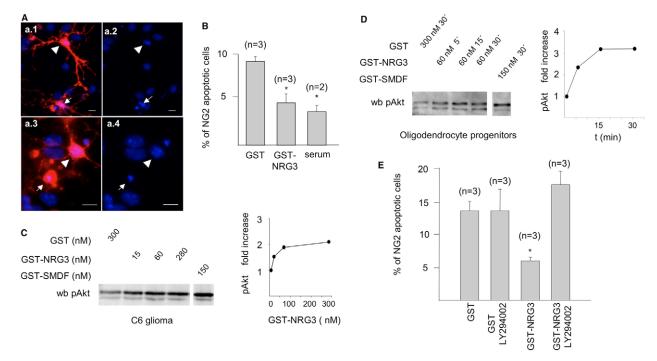
Fig. 8. NRG3 is a survival factor for oligodendrocytes. (A) Serum-starved oligodendrocyte precursor cultures were immunostained with anti-NG2 antibody; a.1 and a.3 overlays; a.2 and a.4 Hoechst dye staining of nuclei. Apoptotic (arrows) and non-apoptotic (arrowheads) cells from 750 to 1500 NG2-positive cells per experiment were counted. Bars, 10 μm. (B) The results of three different experiments are shown. GST-NRG3 (150 nM) decreased apoptosis to similar levels found in non-serum-starved cultures. GST-purified protein was used as control. Data are given as mean ± s.e.m.; the Student's *t*-test was used (*$P \leq 0,015$). (C) Recombinant neuregulin 3 increases the phosphoryaltion of Akt in a dose-dependent way in the rat C6 glioma cell line. Recombinant neuregulin 1 (GST-SMDF) was used as a control. The densitometric quantification of a typical experiment is shown. (D) Time course of Akt phosphorylation in oligodendrocyte precursor cultures. (E) Inhibition of PI 3-kinase pathway with 20 μM LY294002 abrogates the anti-apoptotic effect of recombinant NRG3. Data are given as mean ± s.e.m.; the Student's *t*-test was used, *$P \leq 0.015$.

genes in human and mouse genomes. Finally, the most frequent event is alternative splicing that is genome-specific. This type of splicing involves genes, which contain exons that are only present in the genome of a single species. More than 50% of the spliced genes in humans and mice are spliced in this genome-specific alternative-splicing process (Ahn et al., 2005; Pan et al., 2004; Modrek and Lee, 2003). Interestingly, multiple new transcription initiation sites have been described for the human *NRG1* gene (Steinthorsdottir et al., 2004). Some of the *NRG1* isoforms produced this way are rare brain transcripts that contain internal exons that are not conserved between the human and the mouse genomes. Much less is known about the splicing of the *NRG3* gene. Recently, evidence was presented that multiple NRG3 isoforms are expressed in the mammary gland of mice where they might be involved in mammary-bud induction (Howard et al., 2005). The hFBNRG3 transcript appears to be specific for humans because ESTs with homology to the 5′ region of this mRNA were not found in EST databases from other organisms, including rats and mice. This is consistent with the observation that this sequence has been found in the human genome but not in that of mouse or rat. Interestingly, we found the sequence of exon 2, but not that of exon 3, in the draft of the chimpanzee genome that has recently been published (Chimpanzee Sequencing and Analysis Consortium, 2005). Collectively, our data support the existence of a genome-specific splice isoform of NRG3 that includes newly created exons in the primate genome.

Confocal microscopy and biochemical studies support a plasma membrane localization of the protein. Like other neuregulins, hFBNRG3 is probably a type I transmembrane protein with an extracellular N-terminus and a cytosolic C-terminus. The N-terminal domain contains an EGF-like region and a hydrophobic sequence (V10-Y29). Fusion of an HA-epitope to the N-terminus of the protein reveals that this segment is cleaved, rendering a shorter mature polypeptide. In addition, the extracellular domain contains sites for N- and O-glycosylation. Our data indicate that the protein binds to the N-acetyl-galactosamine-specific SBA lectin, implying that the protein is glycosylated. Furthermore, activity of the protein can be detected in the extracellular milieu, as demonstrated by the erbB4-activating activity of conditioned medium from hFBNRG3-expressing cells. Taken together, these experiments suggest that, like other neuregulins, hFBNRG3 is exported to the cell surface and released into the extracellular medium.

Heterologous expression of the hFBNRG3 clone in mammalian cells can be enhanced by incubation with Lactacystin, a bacterial metabolite with a high specificity for proteasome inhibition (Fenteany et al., 1995). Proteosomal degradation of the hFBNRG3 protein seems to be mediated by ubiquitylation, as suggested by the increase in protein stability and expression following inhibition of ubiquitylation in ts20 cells. However, we could not directly detect ubiquitylation of the hFBNRG3 protein (data not shown), suggesting that its degradation is driven by an interaction with other proteins (Diamonti et al., 2002; Qiu and Goldberg, 2002). In support of this notion, removal of the N-terminal domain spanning residues C3-F55, completely abrogates protein degradation, suggesting the existence of a protein-protein interacting domain in this region.

Accumulating evidence shows that the ubiquitin/proteasome system is involved in numerous aspects of neural cell biology and pathology. In general, the degradation of native proteins has a high energy cost, not only because the elimination of resources, but also, because suppression of natively folded proteins is thermodynamically unfavourable. However, targeted degradation of signalling molecules might provide a mechanism to enable rapid responses to a changing environment. For instance, it has been proposed that regulation of *DCC/Fra* (the receptor for axonal attractant netrin-1) depends on its proteasome-mediated degradation (Bashaw and Goodman, 1999; Johnston and Madura, 2004). Furthermore, synaptic plasticity is controlled by ubiquitin/proteasome degradation (DiAntonio and Hicke, 2004). Studies on long-term potentiation (LTP) have illustrated that PKA, a kinase pivotal in learning, is activated in the absence of cAMP because the regulatory subunits of the enzyme complex are degraded by the proteasome (Johnston and Madura, 2004). These data provide compelling evidence for a central role of the ubiquitin/proteasome pathway in nervous system biology. Thus, regulation of hFBNRG3 stability by proteasomal degradation might be a mechanism used to control the activity of NRG3.

Myelination of the mammalian CNS is a highly regulated process in which ensheathing oligodendrocytes are matched to the number and length of neuronal axons. Oligodendrocytes are postmitotic cells, which develop from oligodendrocyte progenitors that migrate into the developing white matter from their germinal zones in the neuroepithelium. Their development is regulated by cell-intrinsic- as well as cell-extrinsic-factors (Barres and Raff, 1999; Rowitch et al., 2002). Previous work has shown that platelet-derived growth factor (PDGF) secreted by neurons is required for proliferation of oligodendrocyte progenitors during development (Calver et al., 1998; van Heyningen et al., 2001). Overexpression of PDGF-A in transgenic mice under the control of the neuron-specific enolase promoter (*NSE-PDGF-A* mice) caused hyper-proliferation resulting in a large increase in the number of oligodendrocyte progenitors. The extra progenitors generated excess oligodendrocytes but these all died soon after their production, so that a normal number of myelinating oligodendrocytes survived long-term (Calver et al., 1998; van Heyningen et al., 2001). This is presumably because the final number of oligodendrocytes is controlled by an unidentified survival factor generated by neurons. There is some evidence that the survival factor is a member of the neuregulin family (Richardson, 2001; Fernandez et al., 2000; Canoll et al., 1996; Vartanian et al., 1999; Kim et al., 2003). Here, we show that the neuregulin 3 gene product is able to activate pAkt, a protein kinase involved in cell survival by inactivating the transcription factor Bad and caspase 9 (Franke et al., 1997). We also report that recombinant NRG3 promotes survival of serum-starved cultured oligodendrocyte precursors and that the inhibition of the PI 3-kinase pathway with LY294002 blocks the effect of NRG3 on oligodendrocyte-precursor survival. Taken together, our results demonstrate that *NRG3* gene products are survival factors for oligodendrocyte precursors in culture. Ongoing experiments in our laboratory address the question whether NRG3 is a crucial survival factor in vivo.

Materials and Methods
Materials

bovine serum (FBS), antibiotics, Ni-NTA agarose, TA cloning kit and pcDNA3.1/myc-His-A were obtained from Invitrogen. Percoll and nitrocellulose membranes were from Amersham Biosciences. The pcDNA3-erbB4 vector was provided by the Yossef Yarden (Weizmann Institute of Science, Rehovot, Israel). Pfu turbo DNA polymerase was from Stratagene. Rabbit polyclonal anti-myc antibody, mouse monoclonal anti-phosphotyrosine antibody (clone PT-66), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies, sulfo-NHS biotin, streptavidin agarose beads, reduced glutathione (GSH)-agarose beads and bisbenzimide H33258 (Hoechst dye) were obtained from Sigma. Mouse monoclonal anti-ubiquitin antibody was from Zymed Laboratories. Mouse monoclonal anti-HA antibody (clone 12CA5) and lumi-light plus substrate were from Roche. pEYFP-C1 and the anti-green fluorescent protein (GFP) antibody (Living Colors A.v. peptide antibody) were from Clontech. Rabbit polyclonal anti-NG2 antibody was from Chemicon. LY294002 was from Biosource. Rabbit polyclonal p44/42 MAP kinase antibody and pAkt(Ser 473) antibody were from Cell Signaling Technology. Streptavidin Alexa Fluor 546 and other fluorescent conjugated secondary antibodies were from Molecular Probes. Recombinant PDGF AA (PDGFAA) was from Biovision.

Sub-cloning of hFBNRG3 encoding cDNA, and RT-PCR

hFBNRG3-encoding cDNA was amplified from CS0DF004YG18 clone by PCR using Pfu Turbo DNA polymerase with the primers hFBNRG3-*Not*I-sense (5′-CCAGAATGGCGGCCGCGCCAGTAAC) and hFBNRG3-*Not*I-antisense (5′-CTACATCTGCGGCCGCTTGGTCAATG) and cloned in frame, after mutation of the stop codon, with the myc epitope and poly-His tag. To introduce an HA-epitope at the N-terminal end of the hFBNRG3-myc construct, inverse PCR with the PAC I bearing primers HA-hFBNRG3 sense (5′–GCTAGCTTAATTAAGTATCCTTAT-GATGTGCCTGATTATGCGTGTGGTATACCTCCAACCCTTG) and HA-FBNRG3 antisense (5′-CGCGACCTTAATTAACTCCATAATTCTATCAACTGC) were used. PCR products were digested with *Dpn*I and *Pac*I, ligated and transformed. To clone the pECFP-C1 chimeras, the ORF of hFBNRG3 was amplified and cloned in frame using the primers hFBNRG3-*Hin*dIII-sense (5′-CTTTTGAAGCTTTTGATAGAATAATG) and hFBNRG3-*Sal*I-antisense (5′-CTACATGTCGACTCACTTGGTCAATG). Other constructs were obtained using standard molecular biology techniques. All constructs were verified by automatic sequencing. To check the tissue expression pattern of hFBNRG3 in human embryos, seminested PCR was performed on human Multiple Choice First-strand cDNA (Origene). External primers, sense (5′-CATATTTTCCCAGGAGGTGT) and antisense (5′-GTGTTGACATGACATAACCCGTG), were used for the first round PCR. The same antisense primer and an internal sense primer (5′-TCTACCCTGAAGACCCCAGA) were used for the second round of PCR. Amplified products were checked in agarose horizontal gels stained with ethidium bromide, cloned into pCRII 2.1 dual-promoter vector using the Invitrogen TA cloning Kit and sequenced.

Cell line culture and transfections

COS-7 and C6 glioma cells (from ECACC) were cultured in DMEM containing 10% FBS. Rat pheochromocytoma (PC12) cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated bovine serum. Ts20 cells (kindly provided by Alice Dautry-Varsat, Institut Pasteur, Paris) were cultured in DMEM 10% FBS at 30°C unless indicated. Cells were plated on 2-cm²-wells at 250,000 cells/well. After 20-24 hours, cells were transfected with 1 μg of plasmid DNA using lipofectamine 2000 following the manufacturer's recommendations. To prepare conditioned mediums, cells were serum-starved 24 hours post-transfection for 18 hours, and the medium was collected and concentrated using Amicon Ultra-4 filters (Millipore).

Oligodendrocyte progenitor cell culture

Cultures were grown on poly-L-lysine-coated 12 mm glass coverslips as described previously (Lubetzki et al., 1991). Briefly, forebrains were removed from newborn mice, dissociated mechanically in 1× Hanks medium and then digested with trypsin (0.0025%) for 20 minutes at 37°C. After centrifugation and washing, the pellet was passed sequentially through two nylon meshes with the pore size of 150 μm and 63 μm. Cells were layered on a Percoll density-gradient and centrifuged at 20,000 *g* for 45 minutes at 4°C. Glial progenitor cells were carefully pipetted out and washed three times with 1× Hanks. They were the resuspended in DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin. Cells were plated at the density of 5×10⁵ cells per well for 45 minutes to facilitate attachment. Then, 1 ml of Bottenstein and Sato medium (Bottenstein and Sato, 1979) supplemented with 0.5% FBS, 1% antibiotics and recombinant PDGF-AA at 10 ng/ml was added to each well.

Proteasome inhibition experiments

All the inhibitors used were obtained from Sigma. To study the effect of proteasome inhibition on protein stability, hFBNRG3-transfected cells were incubated with 25 μM Lactacystin, 2 μM MG-132 (Z-Leu-Leu-Leu-al) or 20 μM Z-Leu-Leu-Phe-CHO for 18 hours (unless indicated) before lysis. Nontreated cells were incubated with vehicle (DMSO) for the same period of time. After that, cells were submitted to anti-myc immunoblotting or immunocytochemistry as indicated.

Immunoblotting and lectin-binding assays

After transfection, cells were mixed with β-mercaptoethanol containing SDS sample buffer, heated and separated by SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes, blocked with 10% fat-free milk in TBS and incubated with the primary antibody in blocking buffer for 1 hour. Membranes were washed with TBS-Tween (0.3%), incubated with a secondary HRP-conjugated antibody and developed with the ECL plus system. For lectin binding assays, transfected COS-7 cells were lysed with 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.4% sodium deoxycholate, 0.3% SDS and 1% Nonidet P-40, and cell extracts were incubated with 80 μl of SBA- or WGA-agarose lectins and extensively washed with lysis buffer. Immunocomplexes and lectin-bound proteins were analysed by immunoblotting. To rule out nonspecific binding, lectin-bound protein was eluted with 0.3 M galactose.

Isolation of plasma membrane fractions

Crude plasma membranes were essentially prepared as described by Cabedo et al., (Cabedo et al., 2002). Briefly, transfected cells were rinsed with PBS and lysed in hypotonic buffer A (2 mM MgCl₂; 1 mM EDTA; 1% phenylmethylsulfonyl fluoride; 20 mM Hepes pH 7,4). Cell lysates were centrifuged at 9000 *g* 4°C for 10 minutes to prepare low-speed pellets containing the plasma membranes and supernatants, which had the soluble proteins and the remaining membranes. Low-speed pellets were washed first in buffer A supplemented with 1M NaCl and centrifuged at 4°C, second in buffer A for desalting. Final pellet and supernatants were mixed with β-mercaptoethanol-containing SDS sample buffer, and heated and separated by SDS-PAGE.

Biotin labelling of surface proteins

Transfected cells were incubed with 0,1 mg/ml of sulfo-NHS-biotin during 30 minutes at 4°C, and lysed in 10 mM Tris pH 8.8, 150 mM NaCl, 1% sodium deoxycholate, 2% SDS and 2% nonidet P-40. Biotin-conjugated cell-surface proteins were purified with streptavidin agarose, mixed with β-mercaptoethanol-containing sample buffer and separated by SDS-PAGE.

Immunocytochemistry and microscopy

Cells were seeded on poly-L-lysine-coated cover-slips at 50 to 200×10³ cells/well and transfected as indicated previously. At the indicated times, cells were washed with PBS and fixed with 4% paraformaldehyde, blocked with 20% FBS in DMEM (DMEM/FBS) 45 minutes and incubated with primary antibodies in DMEM/FBS 0.1% Triton X-100 at room temperature for 180 minutes. Afterwards, coverslips were washed with PBS and incubated with secondary antibodies in the same medium for 45 minutes. Washes were repeated and cells were incubated with Hoechst dye and mounted on slides. Images of the slides were obtained with a Leica confocal or fluorescence microscope.

Apoptosis and pAkt phoshorylation assays

After 7 days of culture, oligodendrocyte progenitors were serum- and growth-factor-starved and incubated with 150 nM of GST- or GST-NRG3-purified proteins for 48 hours. Then, cells were immunostained with an anti-NG2 antibody, a specific oligodendrocyte-precursor marker, and counter-stained with the nuclear marker Hoechst dye. Numbers of NG2-positive cells with pycnotic nuclei were counted in control and treated cultures. For Akt phosphorylation assays, oligodendrocyte precursor or C6 glioma cells were serum-deprived and cell extracts submitted to anti-pAkt specific antibody immunoblot.

Purification of the recombinant neuregulin from *E. coli*

cDNA encoding the EGF-like domain of NRG3 was cloned in pGEX-4T-1 and transformed in DH5α bacterial cells, which were grown at 37°C until reaching 0.6-0.8 OD. Thereafter, cells were induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) at 0.2 mM for 4 hours and pelleted. The pellet was resuspended in PBS and disrupted by sonication. Triton X-100 was added to reach 1% and the cell extract centrifuged at 3000 *g* for 15 minutes. Recombinant GST-NRG3-EGF-like polypeptide was purified from the supernatant with reduced glutathione (GSH)-agarose beads. After extensive washing, fusion-protein was eluted from the beads with 10 mM GSH in Tris-HCl pH 8. GST was purified using the same procedure. The protein concentration was determined using the method of Bradford (Bradford, 1976).

Tyrosine phosphorylation assay

Neuregulin-induced tyrosine phosphorylation of erbB receptors was carried out by a modified protocol of Ho et al. (Ho et al., 1995). To ensure comparable levels of the receptor per well, pcDNA3-erbB4 transiently transfected COS-7 cells were trypsinised, reseeded and grown to ⩾80% confluence in 24-well plates. Under these conditions the amount of erbB4 per total protein was equal. Thereafter, cells were serum-starved for 18 hours and incubated with concentrated serum-free conditioned medium or recombinant NRG3-EGF-like domain for 5 minutes at 37°C as indicated. Medium was removed and cells were harvested with 100 μl of β-mercaptoethanol-containing SDS sample buffer. Whole-cell extracts were heat-denatured and separated by SDS-PAGE. Nitrocellulose membranes were blocked with 10% fat-

free dried milk in TBS and incubated with the monoclonal anti-phosphotyrosine antibody in blocking buffer for 2-4 hours. Membranes were washed with TBS-Tween (0.3%), incubated with the anti-mouse IgG-HRP-conjugated antibody and developed with the ECLplus system. To verify identical protein load in each lane the membranes were reblotted with anti-PKCα.

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