Neuregulin-1 modulates the differentiation of neural stem cells in vitro through an interaction with the Swi/Snf complex

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ABSTRACT

The neuregulin-1 (Nrg-1) gene is translated into several protein isoforms, which are either secreted or membrane-anchored. In vitro, neural stem cells (NSC) express mainly the cystein-rich-domain NRG (CRD-NRG) isoform, a membrane-anchored type III form. This isoform exhibits a cystein-rich-domain, which constitutes a second transmembrane domain and can be cleaved to release both a signaling EGF-containing domain (ECD) at the cell surface and an intracellular domain (ICD). The main goal of this paper was to determine the exact role of ECD and ICD in NSC survival and differentiation. Using an siRNA approach, we demonstrated that CRD-NRG inhibition was followed by a decrease in NSC proliferation and of neuronal or oligodendroglial differentiation. Moreover, we showed that ICD physically interacted in cultured NSC with BRM and BAF57, two members of the Swi/Snf remodeling complex, and that ICD stimulation of neuronal cell differentiation is dependent on the presence of BAF57.

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Introduction

Among growth factors that could modulate nervous system development, neuregulins (NRG) constitute a specialized family of growth factors, which are better known as regulators of various aspects of the nervous system biology (Adlkofer and Lai, 2000; Leimeroth et al., 2002; Lai and Feng, 2004). Each isoform of neuregulin contains an extracellular EGF-like motif, the hallmark of the Neuregulin family, which consists of four genes: Nrg-1 (Britsch, 2007), Nrg-2 (Carraway et al., 1997; Chang et al., 1997), Nrg-3 (Hijazi et al., 1998; Zhang et al., 1997) and Nrg-4 (Harari et al., 1999). The diversity of the Nrg-1-encoded protein results from an alternative splicing of a primary transcript (Steinthorsdottir et al., 2004). The Nrg-1 protein isoforms have been classified into three groups according to the structure of their N-terminal region: type I, or acetylcholine receptor-inducing activity (ARIA) (Corfas et al., 1993), and neu-differentiation factor (NDF) (Wen et al., 1992; Falls et al., 1993); type II, or glial growth factor 2 (GGF2) (Marchionni et al., 1993; Bermingham-McDonogh et al., 1997) and type III, or cystein-rich-domain (CRD-NRG) (Buonanno and Fischbach, 2001). More recently, it was reported that three lesser isoforms are also expressed; type IV, V and VI, leading to 31 isoforms being detected by analysis of human expressed sequence tags (Tan et al., 2007).

All these isoforms are known to exhibit various activities during nervous system development (Esper et al., 2006). Indeed, Nrg-1-encoded proteins promote proliferation, differentiation and myelination of Schwann cells (Leimeroth et al., 2002) and oligodendrocytes (Raabe et al., 1996). They also promote maturation and survival of astrocytes (Pinkas-Kramarski et al., 1994). In neurons, Nrg-1-encoded neuregulins induce neuronal migration, and selectively increase the expression of several neurotransmitter receptors (Loeb, 2003). They are also implicated in synaptic plasticity (Mei and Xiong, 2008).

The Nrg-1-encoded third group protein, CRD-NRG, is expressed by neural stem cells (NSC) in culture (Calaoa et al., 2001). CRD-NRG is characterized by two transmembrane regions, and a cleavage releasing the extracellular domain (ECD), which is able to recruit ErbB receptors, and the intracellular domain (ICD) (Frenzel and Falls, 2001). However, ICD has also been detected in neuronal nuclei, suggesting a role in neuronal survival as a consequence of a retrograde signaling mechanism (Rao et al., 2003).

We previously demonstrated that the inhibition of NRG using a recombinant and soluble form of ErbB receptor is followed by a decrease in NSC proliferation and survival and by a premature oligodendroglial differentiation in surviving NSC (Calaoa et al., 2001). In this paper, we analyzed the distribution of Nrg-1-encoded isoforms in cultured mouse NSC, as well as, in cultured neurons, astrocytes and oligodendrocytes in order to unravel a possible specific distribution of
ICD and ECD in immature or differentiated cell types. We then investigated the effects of ECD and ICD on proliferation and differentiation of NSC by modulating their expression. Finally, we demonstrated that ICD can interact with BAF57 (or Smarce1) and Brm or (Smarca2), two members of the Swi/Snf remodeling complex.

**Results**

Subcellular distribution of the extra- and the intracellular domain in proliferating and differentiating neural stem cells in culture

In order to determine the subcellular distribution of the extracellular domain (ECD) and the intracellular domain (ICD) of Nrg-1-encoded isoforms, we performed compartmental protein extractions on proliferating and differentiating cultured neural stem cells (NSC), allowing us to extract specifically either cytoplasmic or membranous or nuclei proteins (see Fig. S3). Bearing in mind the fact that CRD-NRG is the prevalent Nrg-1-encoded isoform expressed by proliferating NSC in culture (Calaora et al., 2001) and most available antibodies recognize either α or β Nrg-1-encoded isoforms, we designed a monoclonal antibody against CRD-NRG with an epitope located in the ECD region (a complete description of the D11 monoclonal antibody is available in Fig. S1 and S2).

D11 monoclonal antibody was tested in western blot experiments using compartmental protein extracts. In NSC, a 29 kDa signal was observed in cytoplasmic protein extracts (Fig. 1A), corresponding to the secreted isoform devolved to be released at the cell surface (Loeb et al., 1998). In the membrane protein fraction, we observed a 140 kDa signal corresponding to the full length protein anchored in the membrane (Fig. 1A). An identical profile of the ECD distribution was observed in compartmental extracts prepared using cultured neurons (Fig. 1B). In compartmental protein extracts prepared from cultured astrocytes, the distribution was different, as we observed, in addition to the 29 kDa signal, a 42 kDa signal in the cytoplasmic fraction that could correspond to the NDF isoform (Fig. 1C). Furthermore, the molecular weight of the signal present in the membrane protein fraction was 110 kDa instead of 140 kDa (Fig. 1C), suggesting that D11 monoclonal antibody seemed thus also to recognize type I isoforms. In

![Fig. 1. Distribution at the subcellular level of the extracellular and intracellular domain of Nrg-1. Western blots using D11 monoclonal antibody allowed us to observe the presence of the full length protein (140 kDa) anchored in the membrane and the secreted form (29 kDa) of the extracellular domain in the cytoplasmic protein extracts from neural stem cells (A) and neurons (B). In astrocytes, we observed a full length form in the membrane protein extracts, which exhibited a 110 kDa molecular weight probably corresponding to the Nrg-1 type I membrane-anchored isoform. We also identified a 42 kDa signal in cytoplasmic fraction (NDF isoform), which is the secreted form of Nrg-1 type I (C). In oligodendrocytes, the 140 kDa and 29 kDa were also present as a 120 kDa signal in the cytoplasmic fraction (D). Using an antibody directed against the carboxy-terminal region of Nrg-1, we observed a 55 kDa signal in the membrane and nuclear protein extract fractions from the four types of cell (E).](image-url)
western blot experiments, D11 antibody, in cultured oligodendrocyte protein extracts, recognized signals at 29 kDa in cytoplasm and at 140 kDa in membrane fractions. We also observed a 120 kDa isoform in cytoplasmic extract and this last form has already been suggested as a proprotein form (Frenzel and Falls, 2001) (Fig. 1D). In each cell type, no signal was observed with D11 monoclonal antibody in the nuclear fractions.

We then performed western blot analyses on the same protein extracts using a commercial polyclonal antibody that recognized an epitope present on the carboxyterminal region of the cytoplasmic extremity. In the four types of cell, we observed a 55 kDa signal in both membrane and nuclear fractions (Fig. 1E). Indeed, these signals could result from a post-translational cleavage of the full length isoform. The Nrg-1 intracellular domain was able to be cleaved by a γ-secretase implicated in a Regulated Intramembrane Proteolysis process (Landman and Kim, 2004). This cleavage then allowed the translocation of the intracellular domain into the nucleus. These results also suggest that it would be possible for ICD to be cleaved and translocated into the nucleus, as was previously demonstrated in Bao et al. (2003).

Knock-down of the expression of Nrg-1-encoded isoforms, and of ErbB2 and ErbB4 in cultured NSC

We first validated our transfection protocol by analyzing the percentage of dissociated NSC transfected with 6-Fam-labeled siRNA and observed that around 50% of cells were transfected (data not shown). Three days after various siRNA transfections, we checked, by western blotting, the efficiency of siRNA against their respective targeted protein expression and we observed a decrease in the signal in NSC cultures for Nrg-1 (Fig. 2A), ErbB2 (Fig. 2B) and ErbB4 (Fig. 2C). A densitometric analysis of the signal obtained by western blot shows that the signal was decreased by at least 45% in comparison with the control, a result which corroborates the efficiency of the transfection.

We then analyzed the effect of siRNA on cultured NSC proliferation by quantifying the Ki67-positive cells (Fig. 3A). The transfection with siRNA directed against Nrg-1-encoded isoforms, and ErbB2 and ErbB4 receptors was followed 3 days later by a statistically significant decrease in the number of Ki67-positive cells. There was no difference observed between the knock-down of ErbB2 or of ErbB4 on the number of Ki67-positive NSC. Concomitantly, the inhibition of Nrg-1 and ErbB2 led to a decrease in the proportion of Ki67-positive NSC, which was different from a statistical point of view. Finally, the decrease observed in response to the knock-down of ErbB4 was statistically more important than the decrease observed with the knock-down of Nrg-1. As the inhibition of the expression of Nrg-1, ErbB2 and ErbB4 appeared roughly equivalent (Fig. 2), these observations would suggest that in addition to Nrg-1-encoded isoforms, the proliferation of NSC in culture could also be sustained by another growth factor interacting with the ErbB4 receptor.

Five days after siRNA transfection, we quantified the numbers of differentiated neurons, oligodendrocytes and astrocytes in the siRNA-transfected NSC cultures. Using immunocytofluorescence, neurons, oligodendrocytes and astrocytes were respectively labeled using anti-β III-tubulin, -O4 and -EAAT1 antibodies and were counted. The number of neurons decreased when NSC were transfected with siRNA directed against Nrg-1-encoded isoforms, and ErbB2 and ErbB4 receptors was followed 3 days later by a statistically significant decrease in the number of Ki67-positive cells. There was no difference observed between the knock-down of ErbB2 or of ErbB4 on the number of Ki67-positive NSC. Concomitantly, the inhibition of Nrg-1 and ErbB2 led to a decrease in the proportion of Ki67-positive NSC, which was different from a statistical point of view. Finally, the decrease observed in response to the knock-down of ErbB4 was statistically more important than the decrease observed with the knock-down of Nrg-1. As the inhibition of the expression of Nrg-1, ErbB2 and ErbB4 appeared roughly equivalent (Fig. 2), these observations would suggest that in addition to Nrg-1-encoded isoforms, the proliferation of NSC in culture could also be sustained by another growth factor interacting with the ErbB4 receptor.

that this effect was not related to an activity of the extracellular domain of these isoforms. As we observed a cleavage of CRD-ICD in NSC cultures, we formulated the hypothesis that this differentiation effect is related only to ICD and its translocation into the nucleus of NSC.

**Effects of Nrg-1 type III extracellular and intracellular domain overexpression in cultured NSC**

In order to have a better understanding of the effects of ECD and ICD, we decided to specifically overexpress ECD and ICD in cultured NSC. Three days after transfection with plasmids containing an ECD or ICD sequence, we performed an immunocytofluorescence test using an anti-Ki67 antibody to quantify the NSC proliferation (Fig. 4A). The ECD overexpression did not show any significant change in the number of Ki67-positive NSC compared to the transfected control-NSC. When ICD was overexpressed in NSC, we observed a decrease in the number of Ki67-positive cells, suggesting that ICD could be implicated in the differentiation process. Indeed, 5 days after transfection, analysis by immunocytofluorescence of the number of neurons (βIII-tubulin-positive cells), oligodendrocytes (O4-positive cells) and astrocytes (EAAT1-positive cells) (Figs. 4B–D) showed that overexpression of ICD but not of ECD was followed by an increase in the number of neurons and oligodendrocytes in cultured NSC. No significant changes were observed in the number of astrocytes when ECD or ICD were overexpressed. In order to determine whether the increase in neurons and oligodendrocytes was a consequence of an apoptosis rescue, we performed TUNEL labeling on ICD-transfected NSC and observed no difference between non-transfected and transfected NSC (data not show), indicating thus that ICD does not play a role in the survival of neuron- or oligodendrocyte-differentiating NSC.

**Interactions between the intracellular domains of Nrg-1, BAF57 and Brm**

So far, these results suggested that ICD was responsible for a stimulation of neuronal or oligodendroglial differentiation in NSC cultures. Moreover, these effects were not related to any interaction with ErbB2 or ErbB4 receptors. As we also observed that ICD was also present in the nuclear protein extracts of NSC, we formulated the hypothesis that the effects of ICD could be related to a direct interaction with another protein or other proteins. In order to identify
this (these) protein(s), we decided to use a yeast two-hybrid approach (in collaboration with Hybrigenics°) and identified an interaction of ICD with BAF57 or Smarce1, a nuclear protein of the Swi/Snf chromatin remodeling complex (Luo and Dean, 1999; Yoo and Crabtree, 2009). These protein complexes are able to modulate DNA/nucleosome interactions, allowing or not transcription through an ATPase activity of a motor protein.

In order to confirm these interactions, we first performed co-immunoprecipitation of cultured NSC protein extract using antibodies directed against BAF57 and Nrg-1 but also against Brg1 and Brm, which are the ATPase subunits of the Swi/Snf chromatin remodeling complex. In those conditions, we observed a signal corresponding to Nrg-1 in immunoprecipitations using BAF57 and Brm antibodies but not Brg1 antibody (Fig. 5A). Similarly, we obtained a signal for BAF57 in immunoprecipitated proteins using Nrg-1, Brg1 and Brm antibodies (Fig. 5B). Western blot analysis using Brm antibody showed a signal after immunoprecipitation with Nrg-1 and BAF57 antibodies (Fig. 5C). Finally, we performed a western blot using Brg1 antibody on immunoprecipitates obtained with Nrg-1 and BAF57, indicating an interaction between Brg1 and BAF57 but not with Nrg-1 (Fig. 5D). In conclusion, this co-immunoprecipitation approach revealed that in NSC, Nrg-1 interacts with BAF57 and Brm but not with Brg1.

In order to validate these immunoprecipitation results, we also used a Tap-tag purification approach, in which ICD was cloned into an expression plasmid in a frame with a dual tag (calmodulin binding peptide and streptavidin binding peptide) and then expressed in cultured NSC. Three days after transfection in NSC, proteins were extracted and recombinant ICD was purified by a tandem affinity step in mild conditions, allowing a co-purification of NSC proteins that eventually interacted with ICD. We then performed western blots on the purified proteins using antibodies directed against ICD, BAF57 and Brm. In these three western blots, we obtained a signal corresponding to the expected molecular weight (Fig. 5E) confirming that Nrg-1 intracellular domain, BAF57 and Brm were associated in a complex in NSC. A control Tap-tag approach (transfection of NSC with plasmid containing tags but not the ICD sequence) did not show any Nrg-1, BAF57 or Brm signals in those conditions.

The neuronal differentiation of neural stem cells is related to BAF57 recruitment

Taken together, these observations suggested that ICD stimulated the neuronal differentiation of cultured NSC by recruiting the Swi/Snf complex via interactions with Brm and BAF57. To test such a hypothesis, we used a lentivirus expressing an shRNA directed against BAF57 in transduced NSC. Controls were non-infected cultured NSC and cultured NSC infected with a non-target lentivirus. After infection, NSC were then transfected with the ICD expression plasmid. NSC were allowed to differentiate for 5 days in vitro and we then counted the number of neurons (βIII-tubulin-positive cells) in each condition in vitro through an interaction with the Swi/Snf complex.
(Fig. 6). We were able to observe a decrease in the number of neurons in cultured NSC transduced with a lentivirus expressing shRNA against the expression of BAF57 and this effect was specific, as NSC infected with a non-target lentivirus exhibited the same total number of neurons observed in the non-infected or control condition. Forced ICD expression in cultured NSC was followed by an increase in the number of neurons, as already observed (Fig. 4B). This stimulation of the number of neurons was also observed when cultured NSC were previously infected using a non-target lentivirus. However, the stimulation was completely inhibited when NSC were previously transduced with a lentivirus expressing the shRNA that inhibits BAF57 expression, indicating that the positive effect of ICD on the number of neurons in these conditions depends on a BAF57 interaction.

Discussion

Type III Nrg-1 isoform, or CRD-NRG, is known to have two transmembrane domains and to be able to undergo a proteolytic cleavage, releasing both an extracellular domain (ECD) containing the EGF domain known to interact with ErbB receptors and an intracellular domain or ICD (Frenzel and Falls, 2001). It has been demonstrated that ICD can be translocated to the nucleus of various cell types, including neurons, and its involvement has been suggested in a backward signaling mechanism (Bao et al., 2003). Consequently, the main objective of this study was to characterize Nrg-1 activities in NSC using an siRNA approach and a stimulation of expression of ECD and ICD in neural stem cells. In these conditions, we were able to observe that the inhibition of Nrg-1 expression was followed by a decrease in NSC proliferation, which was less pronounced than the siRNA-mediated inhibition of ErbB4 expression. This observation suggested a possible role of one or more other ErbB4 ligands in the stimulation of NSC proliferation and indicates that Nrg-1 mediates a proliferative signal through ErbB4 receptors. However, ErbB4 inhibition has no effect on neuronal and oligodendroglial differentiation. These observations strengthened the hypothesis of ICD backward signaling in oligodendrocyte and neuronal differentiation. Indeed, we found that siRNA-mediated inhibition of Nrg-1 expression in cultured NSC was followed by a decrease in oligodendrocyte numbers, and a stimulation of expression of ICD but not ECD was found to stimulate oligodendrocyte differentiation. All these observations were thus supportive of the idea that stimulation of oligodendroglial differentiation is mediated by Nrg-1, as has been suggested in a previous study (Calaora et al., 2001). However, this previous study used a soluble and truncated form of recombinant erbB3 receptor to inhibit the Nrg-1 signals in NSC cultures and this approach also inhibited all ligands of the EGF family that recognize erbB3. Here, we demonstrated that Nrg-1 and more precisely, ICD or the cytoplasmic
carboxyterminal extremity of the protein is responsible for this stimulation. More interestingly, we observed that neuronal differentiation was regulated by Nrg-1 and possibly by a similar ICD backward signaling mechanism, as inhibition of Nrg-1 expression but not of ErbB4 or ErbB2 expression was responsible for a decrease in the number of neurons. Moreover, only the stimulation of ICD expression, but not of ECD (part of the Nrg-1 protein isoforms which does interact with ErbB receptors), is responsible for the stimulation of neuronal differentiation.

The hypothesis of the backward signaling of ICD stimulating both neuronal and the oligodendroglial differentiation was supported by the observation that stimulated expression of ICD but not ECD in cultured NSC was followed by an increase in the number of neurons and oligodendrocytes from differentiated NSC. Likewise, the presence of ICD in both membrane and nuclear protein extracts of neurons and of oligodendrocytes was also supportive of a backward signaling of ICD in in vitro neuronal and oligodendroglial differentiation. However, the presence of ICD in nuclear extracts of cultured astrocytes would suggest that ICD would act more as a permissive factor than an inducing factor.

In this study, employing a double-hybrid approach using a mouse E10.5-E12.5 brain cDNA library, we unraveled an interaction between Nrg-1 and BAF57, a member of the Swi/Snf chromatin remodeling complex. Moreover, using both co-immunoprecipitation and Tap-tag, we confirm the interaction between Nrg-1 and BAF57 and we also demonstrated an interaction between Nrg-1 and Brm but not between Nrg-1 and Brg1. Brm and Brg1 are the ATPases that constitute the two principal subunits of the Swi/Snf chromatin remodeling complex. Although these ATPases are highly homologous, it has been demonstrated that they are associated with different promoters during cellular differentiation (Kadom and Emerson, 2003; Bultman et al., 2000).

BRG1 binds to zinc finger protein through a specific domain that is absent in Brm, and Brm interacts with ankyrin repeat proteins, which are critical components of Notch signal transduction.

We demonstrated that ICD stimulating activity of neuronal differentiation was mediated through a recruitment of BAF57, as the inhibition of its expression prevented the ICD effect. This last result implicated the Swi/Snf complex in neuronal differentiation, and it has recently been demonstrated that a switch of the components from this complex is related to the transition of NSC to neurons (Lessaard et al., 2007). In this context, it is possible that ICD participates in such a switch.

In conclusion, we demonstrated that in vitro, the intracellular domain ICD of type III Nrg-1 isoform or CRD-NRG expressed by NSC was implicated in a backward signaling mechanism, allowing a stimulation of neuronal and oligodendroglial differentiation. This effect, appearing as a permissive effect, was related to an interaction with BAF57 and possibly with Brm, both of the Swi/Snf remodeling complex.

**Experimental methods**

**Preparation of NSC culture**

NMRI mice embryos (Harlan°, Kreuzelweg, The Netherlands) were used as the source of neural stem cells and were euthanized by exposure to CO2. They were sent to the University of Ghent for further dissection. Neonatal NMRI mouse cortices were obtained from neonatal NMRI mouse cortices (Invitrogen°) and dissociated in DEM/F12 (Invitrogen°, Brussels, Belgium) (1:1, v/v). The dissociated cells were resuspended in lysis buffer (ActiveMotif°, Rixensart Belgium), and Complete protease inhibitor cocktail (Roche°, Brussels, Belgium). The suspension was plated onto Petri dishes previously coated with 0.1 mg/ml polyornithine and cultured in the same medium. Contaminating astrocyte proliferation was inhibited by two successive cytosine arabinoside Ara-C 10−5 M (Sigma°) treatments in DMEM medium supplemented with 5% horse serum and 10% FCS for 24 h.

**Neuron culture**

E16 mice hippocampi were removed and suspended into 0.25% trypsin and 0.01% DNase (Sigma°) at 3 °C for 20 min. The enzymatic reaction was stopped by adding 10% FCS. The hippocampi were then washed with fresh medium and dissociated in DMEM medium (Invitrogen°) supplemented with glucose 6 g/l, insulin 5 μg/ml (Sigma°), 10% horse serum (Invitrogen°) and 5% FCS. The cell suspension was plated onto Petri dishes previously coated with 0.1 mg/ml polyornithine and cultured in the same medium. Contaminating astrocyte proliferation was inhibited by two successive cytosine arabinoside Ara-C 10−5 M (Sigma°) treatments in DMEM medium supplemented with 5% horse serum and 10% FCS for 24 h.

**Oligodendrocyte culture**

E16 mice cortices were mechanically dissociated and the cell suspension was layered on top of a pre-centrifuged (30 min at 26,000 × g), Percoll density gradient (GE Healthcare®, Roosendaal, The Netherlands 1.04 g/ml) was centrifuged for 15 min at 26,000 × g. Cell debris that remained in the top aqueous phase was discarded and the interphase below the debris and just above the red blood cells was resuspended in PBS–HEPES. The suspension was centrifuged three times (10 min at 400 × g) in PBS–HEPES to eliminate Percoll. The final pellet was resuspended in DMEM supplemented with 10 ng/ml PDGF-AA (Preprotech°, Rocky Hill, New Jersey, USA) and 10 ng/ml bFGF (Sigma°). After 3 days, oligodendrocytes were formed and then plated on T25 plates previously coated with 0.1 mg/ml polyornithine in DMEM supplemented with 0.5% FCS. Oligodendrocytes were collected after 3 days of differentiation.

**Compartmental protein extraction**

Proteins were extracted from mouse E16 striata, floating spheres and cultured neurons, astrocytes and oligodendrocytes. Neurospheres were collected by centrifugation 10 min at 1000 × g and differentiated cells were collected by scraping. Cells were harvested in hypotonic buffer–Tris 20 mM, phosphatase inhibitors 1:20 (ActiveMotif°, Rixensart Belgium), and Complete protease inhibitor cocktail (Roche°, Brussels, Belgium). Extractions were fractioned into soluble proteins (fraction I) and non-soluble proteins (fraction II) by centrifugation at 400 × g for 10 min at 4 °C. Fraction II (which contained nuclear proteins) was resuspended in lysis buffer (Active Motif°) and incubated for 30 min at 4 °C under gentle agitation, then centrifuged at 14,000 × g for 10 min. This step allowed the recovery of nuclear proteins in the supernatant (fraction III). Fraction I was then centrifuged at 125,000 × g for 30 min at 4 °C. The supernatant (fraction IV) contained soluble cytoplasmatic proteins and the pellet (fraction V) contained membrane proteins. Fraction V was resuspended in MBS buffer (MES 25 mM, NaCl 50 mM, 1% Triton X–100). Protein concentrations were quantified by the RC/DC Protein Assay (Bio-Rad°, Nazareth, Belgium) based on the classical method of Lowry.
Western blots

Equivalent amounts of extracted proteins were loaded on each lane of a 10% polyacrylamide gel (NuPAGE, Invitrogen®). After electrophoresis, proteins were transferred onto PVDF membrane (Roche®). After blocking in 0.2% purified casein (Tropix®, Bedford, MA, USA), the membrane was incubated with primary antibody overnight at 4 °C and then with a secondary antibody conjugated with peroxidase for 1 h at room temperature. Immunoreactive signals were visualized by enhanced chemiluminescence (SuperSignal WestPico, Pierce®, Brussels, Belgium). Primary antibodies used were: D11 monoclonal antibody (home made, see Supplementary data); Nrg-1/ICD (Upstate®, Dundee, UK; 1:1000); actin (AbCam®, Cambridge, UK; 1:200); ErbB2 (Santa Cruz®, California, USA; 1:200); ErbB4 (Santa Cruz®; 1:200); BAF57 (Sigma®; 1:1000); Brg1 (Abcam®; 1:1000) and Brm (Abcam®; 1:750). Secondary antibodies conjugated with peroxidase were RG-16 mouse monoclonal anti-rabbit (AbCam®) used at 1:3000, and goat anti-mouse (Sigma®) used at 1:2000.

Transfection with plasmid constructs

The extra- and intracellular domains of CRD-NRG (respectively ECD and ICD) were amplified by PCR (primer sequences displayed in Table 1) – using as a template the previously CRD-NRG cloned cDNA (Birmingham-McDonogh et al., 1997) – and were inserted into pcDNA3.1/V5/His-TOPO (Invitrogen®). Plasmid constructions were sequenced. For the purpose of transfection, neurospheres were mechanically dissociated and individualized NSC were then transfected in suspension using Fugene® HD (Roche®) according to the manufacturer’s instructions. Cells were then plated on laminin-coated coverslips (0.01 mg/ml) for 48–96 h.

Transfection with siRNA

Before transfection, neurospheres were mechanically dissociated and filtered through a 10 μm-pore filter. The filtered cell suspension was transfected with 200 nM of siRNA (sequences displayed in Table 2) (Ambion®, Lennik, Belgium). Transfections were performed using Neofectine® (Ambion®) according to the manufacturer’s instructions. Transfected cells were then plated on laminin-coated (0.01 mg/ml) coverslips for 72–120 h.

Immunocytofluorescence

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and were then permeabilized and blocked (unspecific binding sites) by a 30 min incubation at room temperature in blocking solution (10% donkey serum and 0.3% Triton X-100 in 1×PBS). Cells were then incubated for 1 h at RT with primary antibodies diluted in a 10% donkey serum and 0.1% Triton X-100 in PBS. We used antibodies directed against Ki67 (BD Biosciences®, La Jolla, United States). Both vectors contained two Tap-tags, a streptavidin binding peptide (SBP) and a calmodulin binding peptide (CBP), resulting in the fusion of the Tap-tags to the C-terminus (pCTAP) or N-terminus (pNTAP) of the intracellular domain. Plasmids were prepared using Sigma MidiPrep Kits (Sigma®), checked by sequencing. Dissociated NSC were transfected with these plasmids by electroporation (BTX® ECM 830 cDNA Apparatus, 1 pull, 100 V, 10 ms). Transfected cells were plated on uncoated T75 culture flasks at 2×10^6 cells/20 ml (NUNC®, Langenselbold, Germany) in DEM/F12 (Invitrogen®) supplemented with B27 (Invitrogen®) for 3 days. Cells were then collected and resuspended in lysis buffer provided with the InterPlay TAP Purification Kit (Stratagene®).

Lentiviral transduction

Dissociated and filtered neurospheres were transduced by adding of 100 μl lentivirus expressing shRNA directed against BAF57 (Sigma®) for 1 h at 37 °C. The cell suspension was mixed every 15 min to avoid the formation of aggregates. A proportion of the cells was also transduced with plasmid containing the ICD sequence, as explained previously. Transduced cells were plated on laminin-coated (0.01 mg/ml) coverslips. We used non-target lentivirus (Sigma®) as a negative control and lentivirus turboGFP as a positive control (Sigma®).
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2009.09.003.

References