Neuregulin1 alpha activates migration of neuronal progenitors expressing ErbB4

UNIVERSITÀ DEGLI STUDI DI TORINO

Title: **Neuregulin1 alpha activates migration of neuronal progenitors expressing ErbB4.**

Authors: Benedetta Elena Fornasari[1,2], Marwa El Soury[1,3], Silvia De Marchis[2,4], Isabelle Perroteau[1], Stefano Geuna[1,2] and Giovanna Gambarotta[1*]

1-Department of Clinical and Biological Sciences, University of Torino, Orbassano (Torino), Italy;

2-Neuroscience Institute of the "Cavalieri Ottolenghi" Foundation (NICO), University of Torino, Italy;

3-Department of Molecular Biology, University of Alexandria, Alexandria, Egypt;

4-Department of Life Sciences and Systems Biology, University of Torino, Torino, Italy.

* *Corresponding author*: giovanna.gambarotta@unito.it

Abstract

Deficits in neuronal migration during development in the central nervous system may contribute to psychiatric diseases. The ligand neuregulin1 (NRG1) and its receptor ErbB4 are genes conferring susceptibility to schizophrenia, playing a key role in the control of neuronal migration both during development and adulthood.

Several NRG1 and ErbB4 isoforms were identified, which deeply differ in their characteristics. Here we focused on the four ErbB4 isoforms and the two NRG1 isoforms differing in their EGF-like domain, namely α and β. We hypothesized that these isoforms, which are differently regulated in schizophrenic patients, could play different roles in neuronal migration. Our hypothesis was strengthened by the observation that both NRG1α and NRG1β and the four ErbB4 isoforms are expressed in the medial and lateral ganglionic eminences and in the cortex during development in rat. We analysed *in vitro* the signal transduction pathways activated by the different ErbB4 isoforms following the treatment with soluble recombinant NRG1α or NRG1β and the ability to stimulate migration.

Our data show that two ErbB4 isoforms, namely JMa-cyt2 and JMb-cyt1, following NRG1α and NRG1β treatment, strongly activate AKT phosphorylation, conferring high migratory activity to neuronal progenitors, thus demonstrating that both NRG1α and NRG1β can play a role in neuronal migration.

Keywords:

alternative splicing, neuronal migration, signal transduction, ErbB4, neuregulin1

Abbreviations:

ADAM17, A disintegrin and metalloprotease 17; AKT, cellular homolog of murine thymoma virus akt8 oncogene; ErbB, v-erb-a erythroblastic leukemia viral oncogene homolog; df, degree of freedom; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JMa, juxtamembrane a; JMb, juxtamembrane b; LGE, lateral ganglionic eminence; MAPK, mitogen-activated protein kinase; MGE, medial ganglionic eminence; NRG1, neuregulin1; OB, olfactory bulb; PI3K, phosphatidylinositol-3-kinase; SVZ, subventricular zone; TACE, tumor necrosis factor-α-converting enzyme; TBP, TATA box Binding Protein**.**

Introduction

Neuronal migration represents a critical step in the development of the central nervous system, where neuronal progenitors migrate from their birth site to their final destination (Ghashghaei et al., 2007; Marin et al., 2010).

The tyrosine kinase receptor ErbB4 and one of its ligands, neuregulin1 (NRG1), are involved in the migration of neuronal progenitors from the medial ganglionic eminence (MGE) to the cortex during development (Flames et al., 2004; Lopez-Bendito et al., 2006; Marin, 2013; Marin et al., 2010; Villar-Cervino et al., 2015). Moreover, they also control the migration of subventricular zone (SVZ)-derived neuroblasts toward the olfactory bulb (OB), a process that continues throughout the whole life of the animal (Anton et al., 2004; Birchmeier, 2009; Ghashghaei et al., 2007).

Since NRG1 and its receptors ErbB3 and ErbB4 are genes conferring susceptibility to schizophrenia (Hahn et al., 2006; Iwakura and Nawa, 2013), deeper studies on the NRG-ErbB system could contribute to a better understanding of the role played by this system in both physiological and pathological conditions (Mei and Nave, 2014).

ErbB4 is a tyrosine kinase receptor belonging to the ErbB family. Alternative splicing determines the expression of four different ErbB4 isoforms: JMa-cyt1, JMa-cyt2, JMb-cyt1 and JMb-cyt2 (Mei and Xiong, 2008). The mutually exclusive insertion of exon 15 or 16 determines the expression of JMa (juxtamembrane a) or JMb (juxtamembrane b) isoforms (Mei and Xiong, 2008). Isoforms containing the exon JMa are sensitive to proteolytic cleavage elicited by the metalloprotease ADAM17/TACE (A disintegrin and metalloprotease 17/tumour necrosis factor-α-converting enzyme) which causes the shedding of the extracellular domain, followed by a second cleavage carried out by a presenilin-dependent γ–secretase enzyme (Lee et al., 2002), which releases an intracellular fragment that can be translocated into the nucleus, thus influencing gene

transcription (Sardi et al., 2006). Isoforms containing the exon JMb are uncleavable. Isoforms containing exon 26 are named cyt1, while those missing it are called cyt2. The presence of exon 26 in the intracellular domain of the cyt1 isoform confers to the receptor the ability to bind PI3K (phosphatidylinositol-3-kinase) and to activate the corresponding downstream pathway (Junttila et al., 2000); isoforms containing this exon are also bounded by the E3 ubiquitin ligase and are degraded faster than ErbB4 cyt2 isoforms (Sundvall et al., 2008). The major differences displayed by the four ErbB4 isoforms suggest that they could play different roles by transducing different signals.

ErbB4 can form homodimers or heterodimers with other members of the ErbB family (ErbB1, ErbB2, ErbB3), therefore the signal transduction pathways activated downstream ErbB4 can be deeply influenced by the isoform expressed and by the co-receptor interacting with it (Roskoski, 2013).

In this paper, we investigated the ability of the four ErbB4 isoforms to confer a different migratory activity to neuronal progenitors and to activate the downstream pathways following the stimulation with two NRG1 isoforms. NRG1 gene gives rise to more than 30 different isoforms. The alternative splicing of exons located at the N-terminus allows to divide the NRG1 in 6 different protein types (I-VI): some are synthesized as transmembrane pro-proteins releasing a soluble fragment, others as soluble proteins or transmembrane proteins. NRG1 type I, II, IV, V, VI generate a soluble fragment, while NRG1 type III is a transmembrane isoform (Mei and Xiong, 2008). Alternative splicing of exons located in the C-terminus of the EGF-like domain gives rise to NRG1α and NRG1β isoforms (Edwards and Bottenstein, 2006; Falls, 2003; Wen et al., 1994). In many tissues NRG1β has demonstrated a higher bioactivity compared to NRG1α (Eckert et al., 2009; Wen et al., 1994).

Intriguingly, NRG1β is significantly increased while NRG1α is decreased in the prefrontal cortex of schizophrenic patients (Bernstein and Bogerts, 2013; Bernstein et al., 2013), thus suggesting that the different NRG1 isoforms could play different roles.

It has been demonstrated that ErbB4 expressing neuronal progenitors migrate toward their target, attracted by soluble NRG1, interacting with a permissive cell corridor expressing transmembrane NRG1 (Anton et al., 2004; Flames et al., 2004). However, in these previous studies, the expression of the different ErbB4 isoforms, as well as the expression of NRG1α and NRG1β isoforms and their role in neuronal migration, were not investigated. We previously analysed *in vitro* the role played by the different ErbB4 isoforms in NRG1β-induced migration (Gambarotta et al., 2004), the expression of the different ErbB4 isoforms in the OB and their ability, *in vitro*, to elicit substrate preference (Fregnan et al., 2014). In this study, we further characterized the expression of the different ErbB4 and NRG1 isoforms in the embryonic ganglionic eminences and in the cerebral cortex and we studied the role played by NRG1α and NRG1β in neuronal migration and signal transduction.

Material and Methods

Cell Culture

The ST14A cell line was derived from primary cells dissociated from rat striatal primordia at embryonic day 14 and conditionally immortalized by retroviral transduction of the temperature-sensitive variant of the SV40 large T antigen, as previously described (Cattaneo and Conti, 1998). In this work, we used four previously obtained ST14A stable clones (Gambarotta et al., 2004) each expressing one of the four ErbB4 isoforms: JMa-cyt1 (clone A1.1), JMa-cyt2 (clone A2.1), JMb-cyt1 (clone B1.15) and JMb-cyt2 (clone B2.16). Cells stably transfected with the empty expression vector (pIRESpuro2 vector, Clontech) were used as mock samples. ST14A cells endogenously express ErbB1, ErbB2 and ErbB3. ErbB4-transfected cells were grown as previously described (Gambarotta et al., 2004) at 33°C in medium containing 5 μg/ml puromycin.

Recombinant peptides corresponding to the EGF-like domain of NRG1α (#296-HR) and NRG1β (#396-HB) were purchased from R&D systems.

Ethical standards

Tissue samples (cortex and MGE/LGE, medial/lateral ganglionic eminences) were dissected under the stereomicroscope from four E15 Wistar rats (Harlan). Embryos were obtained by caesarean section from a rat dam deeply anaesthetised by intraperitoneal injection of ketamine (100 mg/kg; Ketavet, Bayer) supplemented by xylazine (5 mg/kg; Rompun, Bayer), and immediately sacrificed by decapitation. All procedures were in accordance with the Council Directive of the European Communities (2010/63/EU), the National Institutes of Health guidelines, and the Italian Law for Care and Use of Experimental Animals (DL26/14) and were approved by the Italian Ministry of Health and

the Bioethical Committee of the University of Torino. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

RNA isolation, cDNA preparation and quantitative real-time PCR

RNA was extracted from MGE/LGE and cortex from four E15 Wistar rats. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Retrotranscription (RT) of 0.75 µg total RNA was carried out in a 25 µl reaction volume containing: 1x RT-Buffer, 0.1 µg/µl bovine serum albumin (BSA), 0.05% Triton, 1 mM dNTPs, 7.5 µM Random Hexamer Primers, 40 U RIBOlock and 200 U RevertAid® Reverse Transcriptase (all RT ingredients were provided by Thermo Scientific). The reaction was performed 10 min at 25°C, 90 min at 42°C, 15 min at 70°C. Quantitative real-time PCR (q-RT-PCR) was carried out using an ABI Prism 7300 (Applied Biosystems) detection system. cDNA was diluted tenfold in nuclease-free water and 5 µl (corresponding to 15ng starting RNA) were analysed in a 20 µl reaction volume, containing 1 x iTaq Universal SYBR Green Supermix (BioRad) and 300 nM forward and reverse primers. Analyses were performed in technical and biological triplicate.

The data from the real-time PCR experiments were analysed using the -ΔΔCt method for the relative quantification. The threshold cycle number (Ct) values were normalized to an endogenous housekeeping gene: TBP (TATA box Binding Protein). As calibrator the CT average of all samples was used. All normalized relative quantitative data are shown as $2^{-\Delta\Delta CT}$. Primers were designed using Annhyb software (http://www.bioinformatics.org/annhyb/) and synthesized by Invitrogen. Primer sequences are reported in Table 1. Predicted amplicon size was validated through capillary electrophoresis analysis of the amplification products (Fragment Analyzer™, Advanced Analytical Technologies).

GENE	Accession Number	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	Amplicon size
ErbB1	NM_031507.1	CACCACGTACCAGATGGATG	CGTAGTTTCTGGGGCATTTC	83
ErbB2	NM_017003	TGACAAGCGCTGTCTGCCG	CTTGTAGTGGGCGCAGGCTG	106
ErbB3	NM_017218.2	CGAGATGGGCAACTCTCAGGC	AGGTTACCCATGACCACCTCACAC	129
ErbB4 JMa	AY375306.1	GGACGGGCCATTCCACTTTACC	CCATGATGACCAGGATGAAGAGCC	90
ErbB4 JMb	AY375308.1	CGGCCTGATGGATAGAACTCCACTG	CCATGATGACCAGGATGAAGAGCC	71
ErbB4 cyt1	AY375306.1	GGAATATTTGGTCCCCCAGGCTTTC	GAGGAGGGCTGTGTCCAATTTCAC	101
ErbB4 cyt2	AY375307.1	GGAATATTTGGTCCCCCAGGCTTTC	GTACACAAACTGATTCCTATTGGAGTCAATTC	91
NRG1α	AF194439	CGACTGGGACCAGCCATCTCATAAAG	TTGCTCCAGTGAATCCAGGTTG	141
NRG1β	AF194438	CGACTGGGACCAGCCATCTCATAAAG	AACGATCACCAGTAAACTCATTTGG	144
soluble NRG1 (type I-II-IV-V)	AF194993	GGCGCAAACACTTCTTCATCCAC	AAGTTTTCTCCTTCTCCGCGCAC	82
transmembrane NRG1 (type III)	AF194439	CCCTGAGGTGAGAACACCCAAGTC	AAGTTTTCTCCTTCTCCGCGCAC	140
TBP	NM_001004198.1	TAAGGCTGGAAGGCCTTGTG	TCCAGGAAATAATTCTGGCTCATAG	68
Dlx2	NM_001191746.1	CAACGAGCCCGACAAGGAAGAC	GAAACTGGAGTAGATGGTGCGTGG	97
Ascl1	NM_022384.1	GAGGGATCCTACGACCCCCTTAGTC	CTCCTGCCATCCTGCTTCCAAAG	117
Pax6	NM_013001.2	CATCGGGTTCCATGTTGGGCC	GGACTGGGGGTTGCATAGGC	114

Table 1. Primers for quantitative real time PCR analysis. The name of the amplified gene or isoform is indicated in the first column, followed by the accession number to the reference sequence. Primer sequences (forward and reverse) are shown in “forward primer” and “reverse primer” columns. In the last column the size (bp) of the amplification product is indicated. A NRG1 isoform diagram in the supplementary information (Fig. S1) shows where the specific primers were designed.

Transwell Assays

Transwell assays were carried out using cell culture inserts (Cat. #353097, BD Falcon) as previously described (Pregno et al., 2011). Briefly, 10^5 cells resuspended in 200µl 2%FBS DMEM were seeded in the upper chamber of the cell culture insert, on a porous

transparent polyethylene terephthalate membrane (8.0µm pore size, 1×10^5 pores/cm^2). The lower chambers contained 800µl 2%FBS DMEM as control condition, or 2%FBS medium added with NRG1α (200ng/ml, 28.5nM) or NRG1β (50ng/ml, 6.24nM). Cells were allowed to migrate 18 hours at 33°C. At the end of the incubation time, inserts were washed with PBS containing calcium and magnesium, and the top side of the membrane was wiped clean with cotton tipped applicators to eliminate unmigrated cells. Migrated cells, present on the lower side of the filter, were fixed in 2% glutaraldehyde for 20 minutes at room temperature, washed with water and then stained with 0.1% crystal violet in 20% methanol. Transwells were photographed (8-bit images, 4x magnification) using a Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Digital Sight DSL1 camera. All conditions were performed in technical and biological triplicate. Four images were analysed for each transwell using the *ImageJ* software and the amount of migrated cells was expressed as the total area of migrated cells (pixel^2). Cells were discriminated by the pores of the transwell membrane by applying a threshold of 300 pixel^2. To take baseline migration into account, values from random migration (in 2% FBS, baseline) were subtracted from chemotactic migration (in 2% FBS + NRG1α or NRG1β) to generate signals of net chemoattraction (Limame et al., 2012).

Protein extraction and Western Blot analysis

Confluent cells grown on 6-cm diameter dishes were serum-starved for 48 hours and then stimulated with NRG1α (200ng/ml, corresponding to 28.5nM), or NRG1β (50ng/ml, corresponding to 6.24nM) in serum free medium. Cells were washed once with warm PBS (33°C) and proteins were extracted by solubilizing cells in 200µl boiling Laemmli buffer (LB, 2.5%SDS, 125mM Tris-HCl, pH6.8). Lysates were collected with a cell scraper, denatured at 100°C for 3 minutes, syringed and spun for 20 minutes at 12000 rpm to

discard cell debris. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma-Aldrich) and equal amounts of proteins (30µg) were loaded into each lane. Proteins were resolved by 8% SDS-PAGE, transferred to a supported nitrocellulose membrane (Biorad #162-0093) and blocked 1 hour at 37°C in 1X TBST containing 5% nonfat milk. Primary antibodies used are: anti-phospho-Erk (1:2000, #9106), anti-phospho-Akt Ser 473 (#4051), anti-phospho-Akt Thr 308 (#4056), anti-total Akt (#9272), anti-total Erk (#9102), anti-phospho-EGF Receptor (#4404), anti-phospho-HER2/ErbB2 (#2247), anti-phospho-HER3/ErbB3 (#4561), anti-phospho-HER4/ErbB4 (#4757) (diluted 1:1000, unless differently specified, and all purchased from Cell Signalling Antibodies); anti-total EGF Receptor (#sc-03), anti-total HER2/ErbB2 (#sc-284), anti-total HER3/ErbB3 (#sc-285), anti-total HER4/ErbB4 (#sc-283) (diluted 1:1000, all purchased from Santa Cruz); anti-β-actin (#A5316, diluted 1:4000, purchased from Sigma); GAPDH (#AM4300, diluted 1:20000, purchased from Ambion). Secondary antibodies used are horseradish peroxidase linked anti-rabbit (#NA934) and anti-mouse (#NA931) diluted 1:40000, purchased from GE Health. All primary antibodies were diluted in 1X TBST containing 5% BSA and 0,02% sodium azide; all secondary antibodies were diluted in 1X TBST containing 1% BSA. Bands were quantified through *Quantity One* software (Biorad).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 22.0 software. All data were expressed as mean + standard error (SEM). Statistical differences between two groups were determined using the two-tailed Student's t-test. Data sets containing more than two groups were tested by applying one-way analysis of variance (ANOVA) with Bonferroni's *post hoc*.

Results

Both NRG1α and NRG1β are expressed in the embryonic medial/lateral ganglionic eminences and cortex

To investigate the expression of the different NRG1 and ErbB4 isoforms in embryonic (E15) rat MGE/LGE and cortex, a quantitative real time PCR (qRT-PCR) analysis was performed (Fig. 1). To validate the accuracy of the dissection, the expression of marker genes for MGE/LGE (Dlx2 e Ascl1) and for the cortex (Pax6) was evaluated (Fig. S2, panel A).

Data show that soluble NRG1 is more highly expressed in the cortex than in the MGE/LGE (Fig. 1A; p=0.0008); conversely, transmembrane NRG1 shows higher expression in the MGE/LGE than in the cortex (Fig. 1B; p=0.0005), in agreement with previous data published by others (Flames et al., 2004). NRG1α expression is similar in the two tissues (Fig. 1C), while NRG1β is more highly expressed in the MGE/LGE region (Fig. 1D; p=0.0005).

The expression of the NRG1 receptor ErbB4 was also analysed; four isoforms of ErbB4 can be produced by alternative splicing: JMa-cyt1, JMa-cyt2, JMb-cyt1 and JMb-cyt2. By qRT-PCR it is not possible to quantify the relative expression of the different exon combinations, because the amplification product would be too long (more than 1200 bp) for quantitative analysis. Therefore, it was only possible to carry out the expression analysis of the single exons (JMa, JMb, cyt1, and cyt2).

Data show that all the analysed ErbB4 exons are more highly expressed in the MGE/LGE than in the cortex (Fig. 1 D-G; JMa p=4.6724E-09, JMb p=7.5843E-05, cyt1 p=1.2795E-07, cyt2 p=1.2244E-07). In these samples we also analysed the expression of ErbB4 co-receptors ErbB1, ErbB2 and ErbB3 at mRNA and protein level: ErbB2 and ErbB3 proteins

are detectable in the MGE/LGE and cortex, while ErbB1 is barely detectable only in the MGE/LGE (Fig. S2, panel B).

NRG1α is able to stimulate the migration of neuronal progenitors stably expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms

As both NRG1α and NRG1β and the four ErbB4 isoforms are expressed in the MGE/LGE/cortex, migration assays were carried out to test whether NRG1α is able to stimulate the migration of neuronal progenitors expressing different ErbB4 isoforms.

The migratory activity of the stable clones derived from the neuronal progenitor cells (ST14A) (Cattaneo and Conti, 1998), each expressing one of the ErbB4 isoform (JMa-cyt1, JMa-cyt2, JMb-cyt1 or JMb-cy2) (Gambarotta et al., 2004) was evaluated through transwell assays following the stimulation with either NRG1α or NRG1β recombinant peptides, corresponding to the EGF-like domain-α and the EGF-like domain-β.

For NRG1β we chose 50ng/ml, which is the concentration used in the previously published experiments; since NRG1α is less bioactive than NRG1β, different ligand concentrations were tested in preliminary assays to identify the best NRG1α concentration generating a detectable ErbB3 phosphorylation (data not shown). Preliminarily tests of NRG1 stimulation on ErbB4 phosphorylation were not conducted, as the aim of the experiment was to investigate the response of the different ErbB4 isoforms to NRG1α and it was impossible to know in advance if the different ErbB4 isoforms were going to have the same response. So, 200ng/ml NRG1α was the chosen concentration for performing the following experiments. Although the stimulation with 200ng/ml NRG1α induced a lower migration and a lower phosphorylation response compared to the stimulation with NRG1β, we chose to use a NRG1α concentration that is not to high, so as to be as close as possible to the physiological conditions; moreover, our aim was to investigate the response of the different

ErbB4 isoforms to NRG1α or NRG1β stimulation, and not to compare the stimulation strength of the two NRG1 isoforms.

Accordingly, the migratory activity of the different stable clones following the treatment with NRG1α (or with NRG1β) was compared to the migratory activity of the corresponding untreated samples (df1=7, df2=16).

Data analysis shows that following the stimulation with NRG1β, all stable clones expressing the different ErbB4 isoforms migrate more highly than control samples (Fig. 2, JMa-cyt1 p=0.0481, JMa-cyt2 p=7.3804E-05, JMb-cyt1 p=0.0002, JMb-cyt2 p=0.0032). On the contrary, following the stimulation with NRG1α, only clones expressing JMa-cyt2 and JMb-cyt1 ErbB4 isoforms show increased migratory activity compared to control samples (JMa-cyt2 p=5.0731E-06, JMb-cyt1 p=8.6344E-06).

Stimulation of the different ErbB4 isoforms with NRG1α and NRG1β

To study the signal transduction pathways activated by NRG1α or NRG1β, neuronal progenitors stably expressing one ErbB4 isoform (JMa-cyt1, JMa-cyt2, JMb-cyt1 or JMb-cy2) (Gambarotta et al., 2004) were starved in serum free medium for 48 hours and then were stimulated for 15 minutes with 200ng/ml NRG1α or 50ng/ml NRG1β. Cells stably transfected with the empty vector were used as mock control.

Experiments were carried out in biological triplicate. Total proteins were extracted, analysed by Western blot and quantified, as shown in Figure 3, where a representative western blot is shown. One-way ANOVA statistical analysis was carried out to compare the protein phosphorylation following the treatment with NRG1α (or with NRG1β) with the corresponding untreated sample (df1=9, df2=20).

In the absence of ligand stimulation, ErbB4 is not phosphorylated. Statistical analysis shows that, after stimulation with NRG1β, ErbB4 is phosphorylated in all stable clones

(Fig. 3, JMa-cyt1 p=7.1520E-07, JMa-cyt2 p=4.2316E-08, JMb-cyt1 p=0.0248, JMb-cyt2 p=1.2633E-06), while after stimulation with NRG1α is only phosphorylated in clones expressing JMa-cyt2 and JMb-cyt1 ErbB4 isoforms (JMa-cyt2 p=0.0468, JMb-cyt1 p=0.0355).

Western blot shows that the expression level of total ErbB4 is not exactly the same in all stable clones. Nevertheless, total ErbB4 quantification (data not shown) shows that there is no correlation between ErbB4 level and phosphorylation following NRG1 stimulation. Indeed, JMb-cyt1 is less expressed, but highly phosphorylated, while JMb-cyt2 is more expressed, but less phosphorylated; JMa-cyt1 and JMb-cyt1, with comparable expression levels, are differently phosphorylated.

ST14A cell line, stably transfected with different ErbB4 isoforms, endogenously express other members of the ErbB family, namely ErbB1, ErbB2 and ErbB3, which could heterodimerize with ErbB4 following ligand stimulation, thus activating different signal transduction pathways. Therefore, the phosphorylation of ErbB1, ErbB2 and ErbB3 following the stimulation with NRG1α or NRG1β was analysed in the neuronal progenitor cells stably expressing different ErbB4 isoforms.

Statistical analysis shows that ErbB3 is phosphorylated after NRG1β stimulation in stable clones expressing JMb-cyt1 and JMb-cyt2 ErbB4 isoforms (Fig. 3, JMb-cyt1 p=0.0294, JMb-cyt2 p=0.0083) and it is not phosphorylated following stimulation with NRG1α. ErbB1 is not phosphorylated, ErbB2 phosphorylation is barely detectable after a very long exposure (data not shown).

To investigate the possible differences among the four ErbB4 isoforms in the signal transduction pathways activated following the stimulation with NRG1α or NRG1β, the phosphorylation of AKT (at Threonine-308 or Serine-473 sites) and of ERK1 and ERK2 were assayed.

Statistical analysis shows that AKT is phosphorylated at Thr^{308} site after the stimulation with NRG1β in all stable clones expressing ErbB4 (Fig. 3, JMa-cyt1 p=0.0020, JMa-cyt2 p=0.0010, JMb-cyt1 p=9.0674E-06, JMb-cyt2 p=0.0037) and after stimulation with NRG1α in stable clones expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms (JMa-cyt2 p=0.0193, JMb-cyt1 p=0.0001).

As concerns AKT-Ser^{473}, statistical analysis shows that AKT is phosphorylated at Ser^{473} site after the stimulation with NRG1β in all stable clones expressing ErbB4 except the clone expressing JMb-cyt2 isoform (Fig. 3, JMa-cyt1 p=0.0263, JMa-cyt2 p=0.0008, JMb-cyt1 p=3.3558E-08), and after stimulation with NRG1α in stable clones expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms (JMa-cyt2 p=0.0200, JMb-cyt1 p=0.0005).

ERK1 and ERK2 were quantified separately, because their regulation could be different. Nevertheless, because their phosphorylation pattern is the same, the graph in Figure 3 shows the sum of ERK1 and ERK2 phosphorylation. Statistical analysis shows that ERK1/2 is phosphorylated in clones stably expressing JMa-cyt1 and JMa-cyt2 ErbB4 isoforms after the stimulation with NRG1β (Fig. 3, JMa-cyt1 p=0.0039, JMa-cyt2 p=0.0462).

Discussion

Deficits in the NRG1/ErbB4 system affect neuronal migration and could account for developmental diseases, such as schizophrenia, for which they are considered susceptibility conferring genes (Chong et al., 2008; Harrison and Law, 2006; Law et al., 2007; Shamir et al., 2012).

When inhibitory interneurons migrate toward the developing cortex, soluble NRG1 isoforms act as chemotactic factors that are expressed by the cortex to attract interneurons expressing ErbB4 (Flames et al., 2004; Ghashghaei et al., 2007). Cortical interneurons are generated from the MGE of the subpallium and migrate through a permissive corridor represented by the LGE, where membrane-bound NRG1 isoforms are expressed (Birchmeier, 2009).

Although many authors described NRG1 as a chemoattractant both in the MGE/LGE/cortex system (Flames et al., 2004; Yau et al., 2003) and in the SVZ/OB system (Anton et al., 2004), it has been recently proposed that NRG1 acts as repellent that funnels interneurons as they migrate from the MGE to cortical destinations (Li et al., 2012). The reasons underlying the discrepancy between the two models are currently unknown. Nevertheless, our previous and current results are consistent with the attractive role of the NRG1 signalling: we previously observed *in vitro* that ErbB4 expression confers to neuronal progenitor cells the ability to migrate toward a soluble NRG1 source (Gambarotta et al., 2004) and to adhere preferentially to a substrate expressing transmembrane NRG1 (Fregnan et al., 2014).

The mRNA expression analysis confirms that soluble NRG1 is more highly expressed in the embryonic cortex, while transmembrane NRG1 is more highly expressed in the MGE/LGE region. NRG1 isoforms can also differ in the C terminus of the EGF-like domain, which can be α or β. Ligand displacement analyses indicated that the structural difference

between α and β significantly affects the affinity of NRG1 to its receptors: the β isoforms bind to mammary cells (expressing NRG1 receptors) with an affinity that is approximately 10-fold higher than that of the α isoforms (Eckert et al., 2009; Wen et al., 1994).

Nevertheless, NRG1α has a wide localization in organs and tissues: it has been found in neuronal cells during development in mouse (Meyer and Birchmeier, 1994) and it is expressed in rat, monkey and human brain (Bernstein et al., 2006). An interesting aspect of NRG1α and NRG1β isoforms relatively to the neuropsychiatric disorders is that their expression pattern changes in opposite directions in the prefrontal cortex of schizophrenic patients: NRG1β expression is increased while NRG1α expression is decreased (Bernstein and Bogerts, 2013; Bernstein et al., 2006).

These data prompted us to further investigate the role of NRG1α: we studied *in vivo* the expression of NRG1α and NRG1β isoforms and we analysed *in vitro* the activity of these isoforms in terms of migration and signal transduction. Our data show that NRG1α is expressed at a comparable level in both MGE/LGE and cortex, while NRG1β is more highly expressed in the MGE/LGE region, thus suggesting that also NRG1α could play a role in the migration from the MGE/LGE to the cortex.

ErbB4 is the tyrosine kinase receptor mediating the NRG1 induced neuronal migration, as demonstrated by the analysis of conditional knockout mice (Anton et al., 2004; Flames et al., 2004; Gerecke et al., 2001). However, the expression and the role of the single ErbB4 isoforms were not investigated in these regions. For this reason, we quantified the expression of JMa, JMb, cyt1 and cyt2 in MGE/LGE and cortex. Our data demonstrate that all the ErbB4 isoforms are expressed in the analysed areas and confirm that ErbB4 is more highly expressed in the MGE/LGE region than in the cortex. Indeed, migrating interneurons express ErbB4 and the differential expression of the ErbB4 isoforms between MGE/LGE and cortex is likely due to the fact that migrating interneurons at E15 are not yet invading the cortex (Yau et al., 2003). The observation that different members of the

NRG1/ErbB4 system are expressed in regions where neuronal migration occurs, induced us to study *in vitro* the migratory behaviour and the activated signal transduction pathways of neuronal progenitors expressing the different ErbB4 isoforms, following the stimulation with two different NRG1 isoforms.

In the MGE/LGE, we observed the expression of ErbB1, ErbB2 and ErbB3, together with ErbB4. The expression of ErbB co-receptors in the MGE/LGE region disagrees with other published data, where it is shown that murine GAD67$^{(+)}$ migrating cortical interneurons express only ErbB4 (Rakic et al., 2015). It is noteworthy that ErbB expression was analysed using end-point PCR on FACS purified murine samples, while our analysis was performed using quantitative RT-PCR, which is a more sensitive technique, on the whole rat MGE/LGE region, and was confirmed also by western blot analysis.

Neuronal progenitor cells (ST14A) stably expressing different ErbB4 isoforms (Gambarotta et al., 2004) were used as *in vitro* model. ST14A cells endogenously express ErbB co-receptors and therefore are a good model to investigate the migratory activity and the signal transduction pathways activated by different ErbB4 isoforms following the stimulation with NRG1. To analyse the activity of NRG1α and NRG1β, recombinant peptides corresponding to the EGF-like domain were used, as recombinant proteins corresponding to the entire extracellular domain of NRG1α were not commercially available.

The migratory activity of stable clones expressing different ErbB4 isoforms was assessed by transwell assay. All stable clones migrate following the stimulation with NRG1β, while only clones expressing JMa-cyt2 and JMb-cyt1 isoforms respond to the stimulation with NRG1α.

In malignant peripheral nerve sheath tumour cells it had been previously shown that NRG1α had no effect on migration (Eckert et al., 2009). Using a different experimental paradigm, we demonstrated that NRG1α is able to stimulate neuronal progenitor migration.

To investigate the signal transduction pathways involved in the migratory activity, stable clones expressing the different ErbB4 isoforms were stimulated with NRG1α or NRG1β and the phosphorylation of ErbB receptors and the activation of PI3K/AKT and MAPK/ERK signal transduction pathways were analysed. In some cases, phosphorylation can be appreciated in the western blot, but the quantitative analysis of the biological triplicate did not give significant results. This discrepancy could be due to the technical limitations of western blot analysis, which is strongly influenced by different factors such as the exposure time, which affects the ratio between the phosphorylated form and the corresponding total protein.

The statistical analysis of ErbB4 phosphorylation shows that all ErbB4 isoforms are significantly phosphorylated following the stimulation with NRG1β, while only JMa-cyt2 and JMb-cyt1 isoforms are phosphorylated following the stimulation with NRG1α. These data show a correlation between NRG1 induced ErbB4 phosphorylation and neuronal progenitor migration.

Although the expression level of total ErbB4 is not exactly the same in all the stable clones, it was found that it does not affect neither ErbB4 phosphorylation nor migration, which seem to be affected only by the expressed ErbB4 isoform type.

Quantitative analysis of ErbB3 phosphorylation exhibits a very high variability. Statistical analysis shows that ErbB3 is significantly phosphorylated in clones expressing JMb isoforms following the stimulation with NRG1β, thus suggesting that ErbB3 might participate in the signal transduction pathways activated by NRG1 treatment. Nevertheless, ErbB3 in the absence of ErbB4 is unable to stimulate cell migration (data not shown).

ErbB1 and ErbB2 are not phosphorylated either in the presence or absence of ErbB4, thus suggesting that they do not heterodimerize with ErbB3 or ErbB4 in this neuronal model.

It has been shown in zebrafish embryos that ERK1 and ERK2 control genes involved in cell migration (Krens et al., 2008a; Krens et al., 2008b). ERK1 and ERK2 in the absence of ErbB4 are not phosphorylated. Statistical analysis shows ERK1 and ERK2 are phosphorylated after the stimulation with NRG1β in clones expressing JMa ErbB4 isoforms.

AKT is an essential mediator of the PI3K signal transduction pathway; it is a serine/threonine kinase playing a critical role in cell growth, differentiation, survival and migration and its activity is tightly regulated through threonine 308 and serine 473 phosphorylation (Hart and Vogt, 2011). Phosphorylation of Thr^{308} is essential for the catalytic activity of AKT and for signalling; this phosphorylation allows the organization of a network of interactions essential for the functioning of the catalytic domain and the formation of the active site (Yang et al., 2002). Phosphorylation of Ser^{473} is required for maximal activation of AKT. Indeed, this phosphorylation leads to further stabilization of the kinase, pushing the equilibrium toward the active conformation and increasing AKT catalytic activity by approximately 10 fold (Alessi et al., 1996).

Our analysis shows that the phosphorylation pattern of AKT-Thr^{308} and AKT-Ser^{473} is the same for all stable clones except for JMb-cyt2.

Data show a strong correlation between ErbB4, AKT-Thr^{308} and AKT-Ser^{473} phosphorylation level and the migratory activity of the clones stably expressing ErbB4.

Indeed, following the stimulation with NRG1α and NRG1β, the two ErbB4 isoforms (JMa-cyt2 and JMb-cyt1) conferring the highest migratory activity show high AKT-Thr^{308} and high AKT-Ser^{473} phosphorylation. In mock cells, which do not migrate following NRG1 stimulation, AKT-Thr^{308} and AKT-Ser^{473} are not phosphorylated. Accordingly, we previously showed that, in the presence of PI3K inhibitors, the migratory activity mediated by ErbB4 following the stimulation with NRG1β is inhibited (Gambarotta et al., 2004).

The ErbB4 isoforms JMa-cyt2 and JMb-cyt1, conferring the highest migratory activity, are deeply different: JMa is susceptible to proteolytic cleavage and can release a cytoplasmic fragment migrating to the nucleus, JMb is resistant to cleavage; cyt1 isoforms contain a domain that mediates the activation of PI3K/AKT pathway (Elenius et al., 1999) and the interaction with E3 ubiquitin ligase (Elenius et al., 1999), cyt2 isoforms lack this domain; both cyt1 and cyt2 isoforms can stimulate MAPK pathway (Sundvall et al., 2008). Therefore, we expect that they activate AKT phosphorylation and cell migration through different ways: cyt1 isoforms can activate PI3K pathway directly, cyt2 isoforms need to heterodimerize with ErbB3, which contains six binding sites for PI3K (Gambarotta et al., 2004; Roskoski, 2013; Vijapurkar et al., 2003). AKT phosphorylation observed following the stimulation with NRG1 could be mediated either by ErbB4 homodimerization (in the case of cyt1 isoforms), or ErbB4-ErbB3 heterodimerization (in the case of cyt1 and cyt2 isoforms).

Conclusion

We showed *in vivo* that different ErbB4 and NRG1 isoforms are expressed in regions where neuronal migration occurs during development. We demonstrated *in vitro* that different ErbB4 and NRG1 isoforms confer to neuronal progenitor cells distinct characteristics and behaviours and that both NRG1α and NRG1β can play a role in signal transduction activation and neuronal migration.

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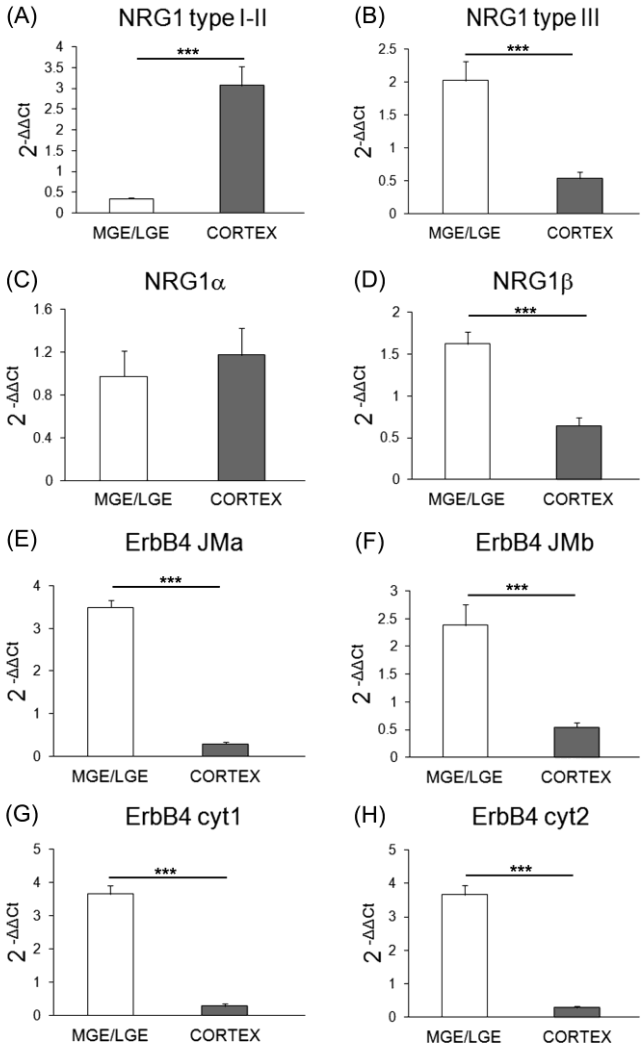
Figure captions:

Fig. 1. Expression analysis of NRG1 and ErbB4 isoforms in embryonic (E15) MGE/LGE and cortex. Analysis of RNA samples obtained from MGE/LGE and cortex of rat embryos at E15. The relative quantification ($-2^{\Delta\Delta Ct}$) of soluble NRG1 (type I-II-IV-V) (A), transmembrane NRG1 (type III) (B), NRG1α (C), NRG1β (D), ErbB4-JMa (E), ErbB4-JMb (F), ErbB4-cyt1 (G) and ErbB4-cyt2 (H) was obtained by quantitative real-time PCR: data were normalized to an endogenous housekeeping gene (TBP). In the absence of a reference calibrator sample, quantitative data were expressed relatively to the average of the two tissue samples. Statistical analysis was carried out using Student's t-Test. Asterisks refer to significant statistical differences, with *** $p \leq 0.001$.

Fig. 2. NRG1α stimulates migration of neuronal progenitors stably expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms. Neuronal progenitors stably expressing different ErbB4 isoforms (JMa-cyt1, JMa-cyt2, JMb-cyt1, JMb-cyt2) were stimulated with NRG1α or NRG1β. The migratory activity was assessed by transwell assay analysis. Migration is expressed in terms of total area of migrating cells ($pixel^2$). Baseline migration values were subtracted from the corresponding chemotactic migration to show the net chemoattraction. Biological triplicate experiments were carried out in technical triplicate. Asterisks refer to one-way ANOVA significant differences between the treated (NRG1α or NRG1β) and the corresponding untreated samples, with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. No statistical differences between samples treated with NRG1α or NRG1β were highlighted, because the concentration of the two ligands is different.

Fig. 3. ErbB3, ErbB4, PI3K and MAPK pathway activation following NRG1α and NRG1β stimulation. Western blot panels showing phosphorylation of ErbB3, ErbB4, AKT

at Thr^{308} or Ser^{473} residues, ERK1 and ERK2 following 15 minutes of stimulation with NRG1α or NRG1β. Experiments were carried out in biological triplicate; the panel shows a western blot representative of the three independent experiments. Molecular mass standards are expressed in kilodaltons (kDa). Protein phosphorylation was normalized to the total amount of the corresponding protein (for each sample the ratio between the phosphorylated band and the total protein was calculated) and the results are shown in the graphics. One-way ANOVA statistical analysis shows significant differences between samples treated with NRG1α or NRG1β and untreated samples (CTRL) (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). No statistical differences between samples treated with NRG1α or NRG1β were highlighted, because the concentration of the two ligands is different.


(A)
NRG1 type I-II
(B)
NRG1 type III
(C)
NRG1α
(D)
NRG1β
(E)
ErbB4 JMa
(F)
ErbB4 JMb
(G)
ErbB4 cyt1
(H)
ErbB4 cyt2
2-ΔΔCt
2 -ΔΔCt
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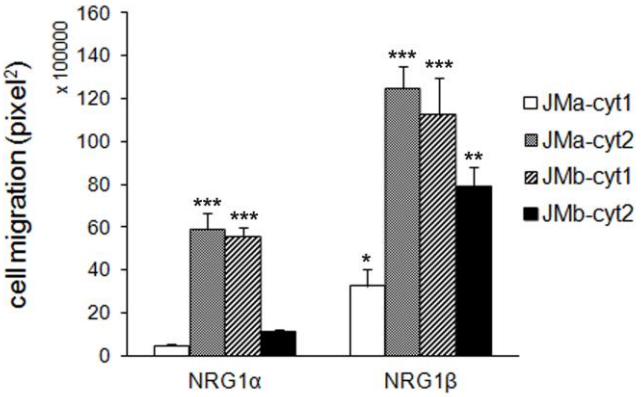
MGE/LGE
CORTEX

Figure 1


cell migration (pixel²)
x 100000
160
140
120
100
80
60
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20
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NRG1α
NRG1β
JMa-cyt1
JMa-cyt2
JMb-cyt1
JMb-cyt2

Figure 2

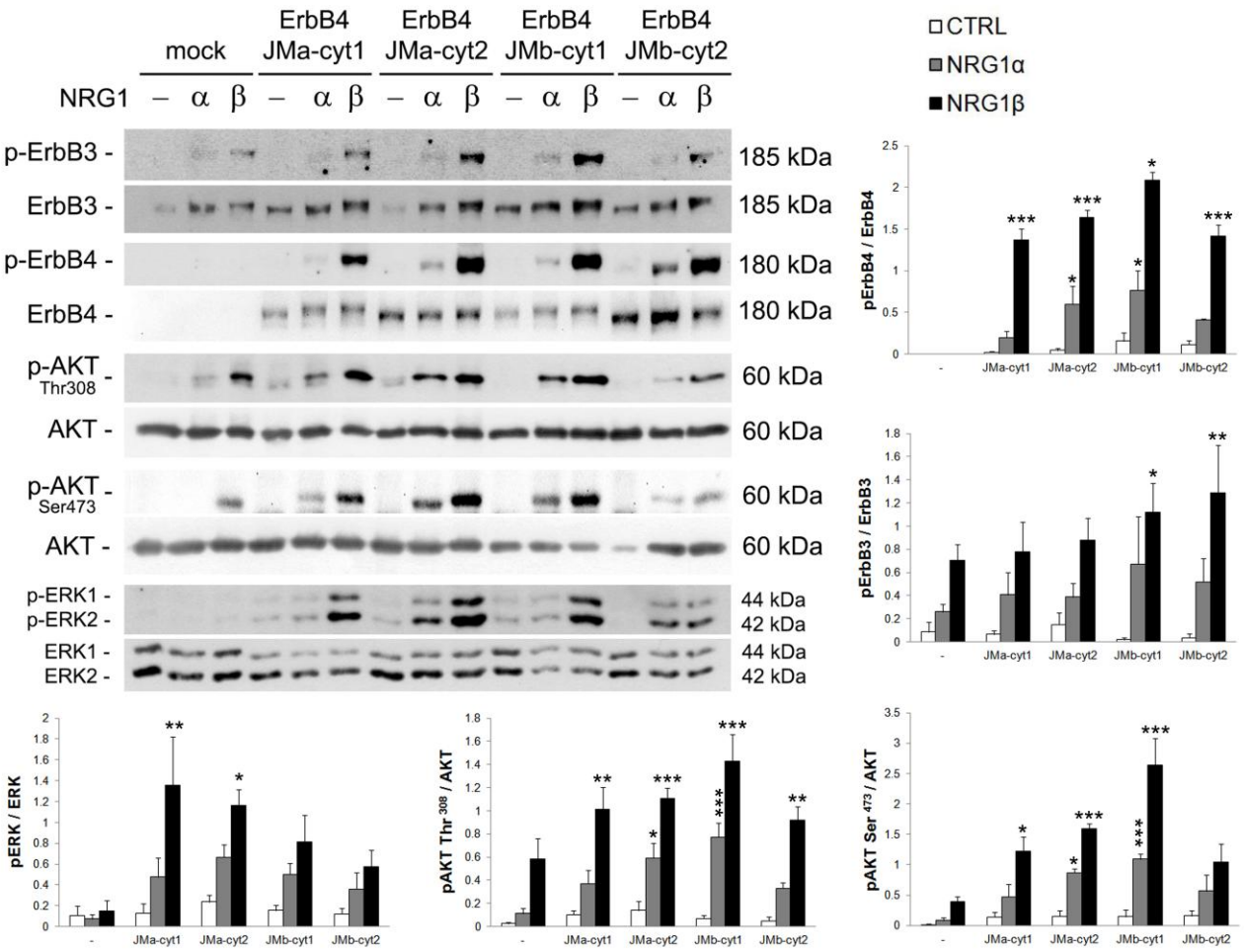
mock
ErbB4 JMa-cyt1
ErbB4 JMa-cyt2
ErbB4 JMb-cyt1
ErbB4 JMb-cyt2
NRG1 – α β – α β – α β – α β – α β
p-ErbB3 -
ErbB3 -
p-ErbB4 -
ErbB4 -
p-AKT Thr308 -
AKT -
p-AKT Ser473 -
AKT -
p-ERK1 -
p-ERK2 -
ERK1 -
ERK2 -
185 kDa
185 kDa
180 kDa
180 kDa
60 kDa
60 kDa
60 kDa
60 kDa
44 kDa
42 kDa
44 kDa
42 kDa
CTRL
NRG1α
NRG1β
pErbB4 / ErbB4
pErbB3 / ErbB3
pERK / ERK
pAKT Thr308 / AKT
pAKT Ser473 / AKT
- JMa-cyt1 JMa-cyt2 JMb-cyt1 JMb-cyt2

Figure 3

Supplementary material

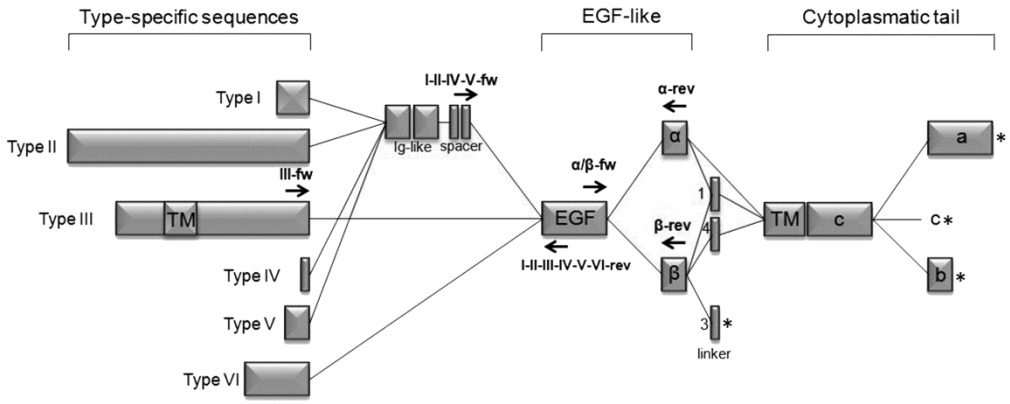

Fig. S1 NRG1 isoform diagram showing where the specific primers were designed. Soluble NRG1 isoforms were amplified with the primers “I-II-IV-V-fw (forward) and “I-II-III-IV-V-VI-rev” (reverse). As can be deduced from the diagram, PCR primers for NRG1 soluble isoforms detect all (I, II, IV and V) soluble isoforms except type VI. Transmembrane NRG1 isoforms were amplified with the primer “III-fw” and the primer “I-II-III-IV-V-VI-rev”. To distinguish between NRG1α and NRG1β, a common forward primer “α/β-fw” and specific reverse primers “α-rev” and “β-rev” were used. The scheme was modified from Mei and Xiong, 2008.

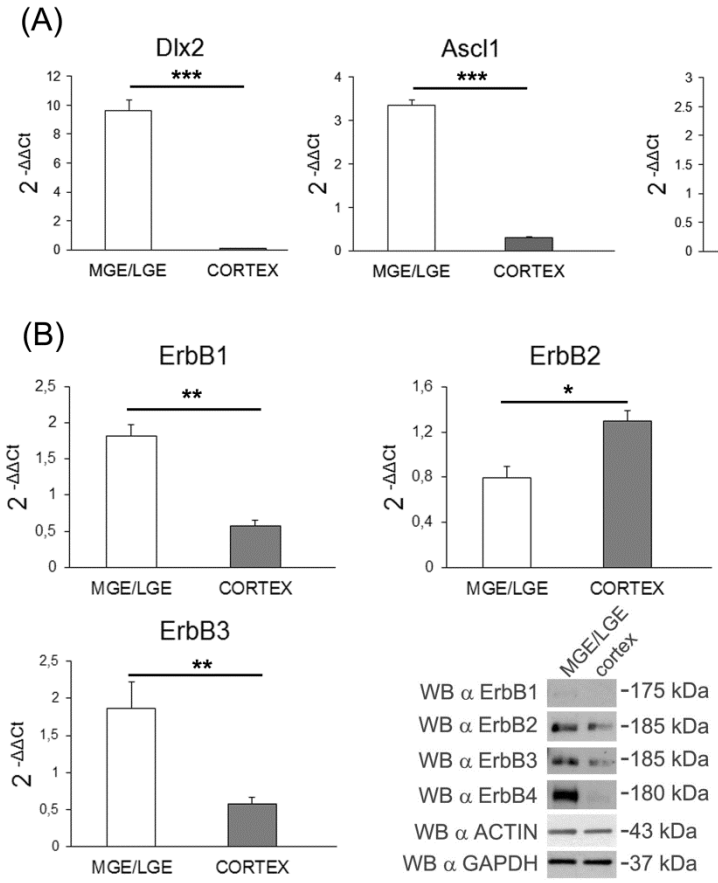

Fig. S2 Expression analysis of marker genes for MGE/LGE and cortex and ErbB4 co-receptors. Analysis of RNA samples obtained from MGE/LGE and cortex of rat embryos at E15. The relative quantification ($-2^{\Delta\Delta Ct}$) of Dlx2, Ascl1, Pax6 (Panel A), ErbB1, ErbB2 and ErbB3 (Panel B) was obtained by quantitative real-time PCR: data were normalized to an endogenous housekeeping gene (TBP). In the absence of a reference calibrator sample, quantitative data were expressed relatively to the average of the two tissue samples. Statistical analysis was carried out using Student's t-Test. Data analysis shows that Dlx2, AscII, ErbB1, ErbB3 are more highly expressed in MGE/LGE samples, while Pax6 and ErbB2 are more highly expressed in cortex samples (Dlx2 p=0.0009, AscII p=0.0001, ErbB1 p=0.0075, ErbB3 p=0.0087, Pax6 p=3.8364E-05, ErbB2 p=0.0285). Western blot (in Panel B) shows ErbB1, ErbB2, ErbB3, ErbB4 protein expression in MGE/LGE and cortex regions. Housekeeping proteins actin and GAPDH were used as a loading control. Molecular mass standards are expressed in kilodaltons (kDa).