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Abbreviations

ADAM 17, A Disintegrin And Metalloproteinase; CRD, cysteine-rich domain; DMEM, Dulbecco's Modified Eagle Medium; EGFR, Epidermal Growth Factor Receptor; FBS, Foetal Bovine Serum; LGE, Lateral Ganglionic Eminence; MGE, Medial Ganglionic Eminence; NRG1, Neuregulin 1; OB, Olfactory Bulb; PBS, Phosphate Buffered Saline; PI3K, Phosphatidylinositol 3-Kinase; q-RT-PCR, quantitative-real time PCR; RFP, Red Fluorescence Protein; RMS, Rostral Migratory Stream; RT, Reverse Transcriptase; SEM, Standard Error of the Mean; SMDF, Sensory and Motorneuron-Derived Factor; SVZ, Subventricular Zone; TACE, Tumor-necrosis-factor- α -Converting Enzyme; TM, transmembrane domain; TK, tyrosine kinase domain.

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Abstract

Soluble and transmembrane neuregulin1 isoforms can act as short and long range attractants for migration of cortical and olfactory interneurons expressing ErbB4, a tyrosine kinase receptor whose characteristics are strongly affected by alternative splicing. Here we investigated the expression of the four ErbB4 isoforms and we found that all of them are expressed by neural progenitor cells migrating from the subventricular zone toward the olfactory bulb, through the rostral migratory stream. We quantified the absolute expression of the different ErbB4 isoforms and we found that all of them are highly expressed in the regions characterized by high interneuron migration, whereas in the olfactory bulb regions - where migration stops - ErbB4 isoforms containing the exon JMb and lacking the exon cyt1 ("cyt2 isoforms") are more expressed. Indeed, we previously demonstrated that neural progenitor cells stably expressing ErbB4-JMb-cyt2 have a very low migratory activity. To find out whether the different ErbB4 isoforms confer distinct adhesion preference for neuregulin 1, cells expressing them were tested *in vitro* in a stripe choice assay. We demonstrate that each of the four ErbB4 isoforms is able to confer to neural progenitor cells an adhesion preference for cells expressing the transmembrane neuregulin1 type III.

Key words: alternative splicing, adhesion preference, ErbB4, neuregulin1, neural progenitor cells

Introduction

ErbB4 is a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family which includes four members; receptor-ligand interactions result in activation of intracellular signaling cascades and induction of cellular responses including proliferation, migration, differentiation, and survival or apoptosis [1,2].

Among ErbB4 ligands are neuregulins (NRG), which comprise a large family of EGF-like signaling molecules involved in cell-cell communication during development and disease. Many different isoforms of the NRG1 gene are synthesized, which differ in their tissue-specific expression pattern and their biological activities, thereby contributing to the great diversity of the functions of NRG1 [3]. Soluble NRG1 isoforms are involved in indirect interactions among cells (paracrine or autocrine), transmembrane isoforms mediate direct cell-cell interactions (juxtacrine interactions).

Four ErbB4 isoforms derived from alternative splicing were described [3] that differ from each other, both structurally and functionally (see Figure 1).

The presence in the extracellular juxtamembrane region of the exon JMa (as an alternative to the exon JMb) confers susceptibility to the proteolytic cleavage by the metalloprotease A Disintegrin And Metalloproteinase/Tumor-necrosis-factor- α -Converting Enzyme (ADAM 17/TACE), which results in the release of the extracellular domain ("ectodomain shedding"); after that, the membrane anchored receptor becomes the substrate of a γ -secretase preseniline-dependent protease

that releases a cytoplasmic domain of ErbB4, which can shift to the nucleus and interfere with transcriptional regulation [4].

The presence in the cytoplasmic domain of the exon cyt 1 (the lack of which is called "isoform cyt2") confers to the receptor the capability to interact with the signal transducer phosphatidylinositol 3-kinase (PI3K) - activating the downstream pathway - and with the WW domain-containing E3 ubiquitin ligase [5-7] - activating the ubiquitin mediated receptor degradation.

The existence of four different isoforms - whose expression is regulated spatially and temporally - evokes functional differences.

It has been shown that ErbB4 is involved in neuronal migration during development [8]: a subpopulation of medial ganglionic eminence (MGE) derived interneurons, migrating toward the cortex, expresses ErbB4 [9]. In the lateral ganglionic eminence (LGE) these cells move through a corridor that expresses the transmembrane NRG1-type III isoform, which acts as a permissive guidance cue for their migration.

ErbB4 is also expressed - during development and also in the adult life - by newly generated neurons tangentially migrating from the subventricular zone (SVZ) to the olfactory bulb (OB) through the rostral migratory stream (RMS).

Transmembrane NRG1-type III isoform is expressed in the regions surrounding the RMS, including the anterior olfactory nucleus, suggesting that it may provide a permissive substratum for neuroblast migration toward the OB [10]. NRG1-type III is also expressed in the internal granule cell layer, as well as in the mitral cell and glomerular layers of the OB [10].

Accordingly to these hypotheses, it was shown that ErbB4 expressing dissociated MGE interneurons [9] and SVZ derived explants [10] display adhesion preference for cells expressing transmembrane NRG1-type III. Until now, analysis of ErbB4 function in neuronal migration and preferential adhesion has been carried out using tools that do not differentiate among the individual ErbB4 isoforms and the contribution of each ErbB4 isoform to the adhesion preference has not been studied. Therefore, we investigated which isoforms are expressed by OB interneurons and whether cells expressing different ErbB4 isoforms display distinct adhesion preference.

Methods

Cell Culture and Reagents.

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated. COS7 cells were purchased from and maintained as recommended by American Type Culture Collection. ST14A is a neural progenitor cell line kindly provided by prof. Elena Cattaneo, derived from primary cells dissociated from embryonic day 14 rat striatal primordial; cells were cultured how previously described [11]. ST14A cells express endogenously ErbB1, ErbB2, ErbB3. Cell clones stably expressing different ErbB4 isoforms or NRG1-typeIII- β 3 were grown in medium containing 5 μ g/ml puromycin. Restriction and modification enzymes were purchased from New England Biolabs.

RNA extraction

Entire OB from adult mice and microdissection samples (from SVZ, posterior RMS, anterior RMS and surrounding OB, see Figure 2A) from six CD1 postnatal day 5 (p5) mice were obtained as previously described [12]. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA from six microdissection samples was pooled to obtain a suitable amount of RNA for quantitative real time PCR analysis.

Reverse transcriptase and quantitative real time PCR analysis

0.5 μ g total RNA were subjected to a reverse transcriptase (RT) reaction in 20 μ l reaction volume containing: 1x RT-Buffer (Invitrogen), 0.1 μ g/ μ l bovine serum

albumin (BSA), 0,5 μ M dNTPs; 7.5 μ M random decamers (Invitrogen); 40 U RIBOlock (Fermentas) and 200 U SuperScript III Reverse Transcriptase (Invitrogen). The reaction was performed for 10 min at 25°C, 90 min at 50°C, 15 min at 70°C.

Quantitative real-time PCR was performed using an ABI Prism 7300 (Applied Biosystems) detection system. cDNA (corresponding to 10ng starting RNA) was analyzed in a total volume of 20 μ l containing 1 x iTaq Universal SYBR Green Supermix (BioRad) and 300 nM forward and reverse primer (see Table, Supplemental Digital Content 1). For each cDNA sample, three technical replicates were averaged and dissociation curves were routinely performed to check for the presence of a single peak corresponding to the required amplicon. For the absolute quantification of the different ErbB4 isoforms, standard samples containing 10, 10², 10³, 10⁴, 10⁵, 10⁶ copies of cDNAs expressing the different ErbB4 isoforms were used. All data were normalized to the relative amount of the starting material of the different samples. The geometric average of the Ct of two endogenous housekeeping genes (Ubiquitin C and TATA box binding protein) was used to calculate the relative starting material. All primers (Table 1) were designed using Annhyb software (<http://www.bioinformatics.org/annhyb/>) and synthesized by Invitrogen.

Expression vector for neuregulin1-typIII- β 3.

We cloned and sequenced the rat transmembrane NRG1-typIII- β 3 cDNA (GenBank ID: DQ176766) as previously described [13] To generate stable cell

lines expressing NRG1-typeIII- β 3, the cDNA was subcloned from pCR®-BluntII-TOPO® vector into the EcoRI site of the expression vector pIRES-puro2 (Clontech); the orientation of the cDNA in the vector (sense or antisense) was detected by BamHI restriction enzyme digestion.

Stable and transient transfections.

For stable and transient transfections, cells were transfected with plasmidic DNA (10 μ g) and Lipofectamine 2000 (10 μ l) (Invitrogen) in Opti-MEM I (Invitrogen) as previously described [14]. For stable transfections, puromycin was added 48 hrs after transfection and antibiotic resistant clones were recovered and screened for NRG1-typeIII- β 3 expression by Western blotting. Cells transiently transfected with expression vectors for the red fluorescence protein and ErbB4 isoforms were used for the stripe choice assay 48 hours after transfection.

Total Protein Extraction and western blot analysis.

Total proteins were extracted and analyzed as previously described [14]. A polyclonal primary antibody anti-N-terminus of NRG1-type III (# AB5551, 1:500 working dilution/w.d., Chemicon) and the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (#NA934, 1:10.000 w.d., Amersham Biosciences) were used.

Stripe Choice Assay

Stripe choice assay was performed by partially modifying the protocol described by others [9], who demonstrated that transmembrane NRG1 is a permissive substratum for ErbB4 expressing cells. Briefly (see Figure 2D), ST14A cells stably expressing transmembrane NRG1-tyrIII- β 3 were plated in a 6 cm diameter plate, grown at approximately 95% confluence and stained with 10 μ M Cell Tracker Green (Molecular Probes) in serum free medium (Dulbecco's modified eagle medium/DMEM) for 40 min, then washed with phosphate buffered saline (PBS). To produce the stripes, green cells were removed with a pipette tip (one line every 2 mm, about 20 stripes/plate). $1,8 \times 10^6$ control cells/well (stably transfected with the empty vector) were plated on top. Plates were incubated for 40 min to allow cell attachment to the empty stripes. The cell excess was washed out with three rinses of PBS, then DMEM containing 10% foetal bovin serum (FBS) was added.

The same day, previously obtained [14] ST14A stable clones - each expressing one of the four ErbB4 isoforms and *mock* cells stably transfected with the empty vector - were transiently transfected with pDsRed1-N1 (Clontech) to obtain red fluorescent protein (RFP) expressing cells. When using COS7 cells, they were transiently co-transfected with 10 μ g expression vector for one of the four ErbB4 isoforms and 1 μ g pDsRed1-N1).

24 hrs later, cells in the stripes reached the confluence; RFP-expressing cells (5×10^5 cells/plate) were plated on top of cell stripes (green=NRG1 expressing; not colored=mock). Analysis was performed 24 hrs later. Cells were fixed 40 min with 4% paraformaldehyde (PAF), then washed with PBS and stored at 4°C in PBS,

with 0,02% sodium azide. For each plate, at least 15 stripes were photographed, using an Olympus IX50 inverted microscope equipped with a Cool SNAP-Pro CCD camera, paying attention to include in each photo a green labeled stripe and a not colored stripe. Images were edited with Image Pro-Plus software; for each photo the green area, the not colored area, the number of red cells on the green area and the number of red cells on the not colored area were measured. For each clone expressing an ErbB4 isoform, the number of red cells/green area with the number of red cells/not colored area were compared. At least three independent experiments (biological triplicate) were carried out.

Statistical analyses

Data are presented as mean + standard error of the mean (SEM). All data were statistically analyzed by One-Way ANOVA and post hoc analysis by means of Bonferroni test (SPSS software).

Results

Different ErbB4 isoforms are expressed by neural progenitor cells tangentially migrating toward the olfactory bulb through the rostral migratory stream

Newly generated neurons tangentially migrating from the SVZ to the OB through the RMS express ErbB4, as previously demonstrated by Anton and colleagues, who observed a near perfect colocalization of ErbB4 and the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), expressed by migrating neuroblasts in the RMS [10].

To study in more detail the role played by ErbB4 in neuroblast migration, we identified the ErbB4 isoforms expressed in the SVZ-RMS-OB region.

By quantitative real time PCR (q-RT-PCR) analysis it is possible to determine the expression level of the single exons (JMa or JMb, cyt1 or cyt2), not the expression level of the different isoforms obtained by alternative splicing (JMa-cyt1, JMa-cyt2, JMb-cyt1, or JMb-cyt2, see Figure 1), because the amplification product from JM exon to cyt1 exon is too long (> 1200 bp) for q-RT-PCR. Indeed, by preliminary assays, we observed the expression of the four isoforms in the SVZ-RMS-OB region, but these data were only qualitative (data not shown).

Primers specific for each single exon were designed (JMa or JMb, cyt1 or cyt2, see Figure 1 and Table 1); for the absolute quantification calibration curve, serial dilutions of plasmids containing cDNAs coding for the four ErbB4 isoforms were used [14].

Coronal slices were collected from 6 postnatal day 5 (p5) CD1 mice and microdissected areas were pooled to obtain an adequate amount of RNA for

expression analysis. Microdissected areas corresponded to the following forebrain regions: the SVZ of the lateral ventricle (SVZ), the posterior part of the RMS (pRMS), the anterior part of the RMS (aRMS) and the OB layers surrounding the anterior RMS (sOB) (Figure 2A).

Data shown here are representative of six animals pooled; however, because only one q-RT-PCR reaction was feasible, it was not possible to perform statistical analysis comparing the expression of all different isoforms in all different areas. Nevertheless, statistical analyses comparing all data relatively to all microareas (adult OB, p5 SVZ, p5 pRMS p5 aRMS, p5 sOB, n=4) and all data relatively to all isoforms (JMa, JMb, cyt1, cyt2, n=5) were carried out. Data analyses show a significant difference between the aRMS microarea (where the transcript copy number is 3,6 higher) and the other areas (with adult OB, $p=0,002$; with p5 SVZ, $p= 0,001$; with p5 pRM, $p=0,000$; with p5 sOB, $p=0,025$). Expression analysis shows that the four exons are all expressed in the SVZ and in the RMS at quite similar levels. Interestingly, in the OB region surrounding the aRMS, containing differentiated interneurons, a strong difference between JMa and JMb isoforms can be observed, suggesting that differentiated interneurons - that reached their target - express higher levels of JMb isoforms, while JMa and JMb isoforms are expressed at similar level by migrating neurons. Accordingly, in the whole adult OB, where the number of migrating neurons is lower than at p5, the number of JMb transcripts is higher. Also, differentiated interneurons seem to express more cyt2 isoforms than cyt1.

Neural progenitor cells expressing different ErbB4 isoforms adhere preferentially on cells expressing transmembrane NRG1-type III

We already demonstrated that JMa-cyt2 and JMb-cyt1 isoforms confer to neural progenitor cells an higher migratory activity than JMa-cyt1 and JMb-cyt2 isoforms [14]. Other authors demonstrated that ErbB4 expressing interneurons adhere preferentially on transmembrane NRG1-type III expressing cells [9,10], whereas ErbB4 conditional knock out do not. To investigate whether the different ErbB4 isoforms confer distinct adhesion preference, we carried out a *Stripe Choice Assay* (see Figure 3B) using neural progenitor cells stably expressing the transmembrane NRG1-typeIII- β 3.

NRG1-type III represents a subgroup of NRG1 isoforms characterized by the presence of an N-terminal cystein rich domain containing an additional transmembrane domain. Different exons located in the C terminal domain give rise - through alternative splicing - to different isoforms. Many of them contain a second transmembrane domain and need a proteolytic cleavage to expose the EGF-like domain toward the external environment and interact with the corresponding receptors (Figure 1).

The neural progenitor cell line ST14A [11] was transfected to obtain cells stably expressing the transmembrane typeIII- β 3 isoform of the ligand NRG1 (Figure 3A) [15]. This isoform has been chosen because it is a transmembrane isoform that does not need a proteolytic cleavage to be active: it contains a transmembrane

domain only and it exposes the EGF-like domain toward the external environment; therefore, it is ready to interact with the corresponding receptor. Two independent clones for each ErbB4 isoform - previously obtained by stable transfection and characterized [14] or *mock* cells (stably transfected with the empty vector) were transiently transfected to obtain Red Fluorescent Protein (RFP) expressing cells.

Alternative stripes of ST14A cells stably expressing transmembrane NRG1-typelll- β 3 (labeled with "cell tracker green") and ST14A cells stably transfected with the empty vector (*CTR*, uncolored cells) were obtained and RFP-ErbB4 expressing cells (or RFP-*mock* cells) were plated on top of the stripes; their final location was observed 24 hrs later.

For each clone stably expressing an ErbB4 isoform, we compared the number of red cells adhering on NRG1-typelll-expressing cells with the number of red cells adhering on control cells and we expressed these numbers as percentages (Figure 3 C). For each ErbB4 isoform, two different stable clones were analyzed in independent experiments (n=4 independent experiments, 15 green and 15 uncolored fields examined for each experiment).

Our data show that cells expressing different ErbB4 isoforms prefer to adhere on NRG1-typelll- β 3 expressing cells (for JMa-cyt1 $60,5\% \pm 2,9\%$, $p=0,000$; for JMb-cyt1 $60,4\% \pm 1,4\%$, $p=0,000$; for JMa-cyt2 $65,7\% \pm 4,1\%$, $p=0,000$; for JMb-cyt2 $64,9\% \pm 5,4\%$, $p=0,000$). Mock cells, although expressing the NRG1 receptor ErbB3, together with ErbB1 and ErbB2 co-receptors, do not display a significant adhesion preference ($54,8\% \pm 5,0\%$, $p=0,394$) suggesting that ErbB4 is the

NRG1 receptor that mediates the adhesion preference toward transmembrane NRG1-type III.

COS7 cells expressing different ErbB4 isoforms display adhesion preference toward transmembrane NRG1 expressing cells

To test the adhesion preference elicited by the different ErbB4 isoforms in a cell context not expressing ErbB3, we performed the stripe choice assay in a different cell model: COS7 cells transiently transfected with the different ErbB4 isoforms, plated on stripes of ST14A cells expressing transmembrane NRG1-typeIII alternated to control cells (n=3 independent experiments, 15 green and 15 non-colored fields examined per experiment).

Our data show that also COS7 cells, transiently expressing different ErbB4 isoforms, prefer to adhere on NRG1-typeIII- β 3 expressing cells (for JMa-cyt1 $57,5\% \pm 0,5\%$, $p=0,002$; for JMb-cyt1 $56,4\% \pm 0,2\%$, $p=0,006$; for JMa-cyt2 $57,2\% \pm 0,9\%$, $p=0,002$, for JMb-cyt2 $57,2\% \pm 1,7\%$, $p=0,002$) (Figure 3D). Mock COS7 cells - not expressing ErbB4 - do not display adhesion preference ($50,3\% \pm 4\%$, $p=1,000$).

Discussion

NRG1-ErbB4 interactions mediate short and long range attraction for tangentially migrating neurons: ErbB4 is expressed by interneurons migrating from the MGE towards the cortex [9] and by neuroblast progenitor cells that migrate from the SVZ, through the RMS, into the OB [10]. In both of these neuronal migration systems, neurons expressing ErbB4 reach their target passing through a permissive corridor that expresses transmembrane NRG1-typeIII (that interacts juxtacrine with ErbB4), stimulated and attracted by soluble NRG1-typeI or -typeII (that mediates paracrine interactions).

Until now, the study of the role of ErbB4 in neuronal migration and adhesion preference has been focused on the analysis of ErbB4 deficient mice, without considering the contribute of the different ErbB4 isoforms.

A growing number of data emphasize that ErbB4 and NRG1 may confer susceptibility to some forms of schizophrenia [16-20]. Therefore, it is informative to test the possible correlation between the expression of particular ErbB4 isoforms and deficits in migration or adhesion. Actually, a prominent expression of the JMa-cyt1 isoform was observed in schizophrenic patients [21] and we observed *in vitro* that this ErbB4 isoform confers to neural progenitor cells a low migratory activity [14].

Patients with schizophrenia are characterized by a reduced OB volume, exhibiting both structural and functional olfactory deficits [22,23] suggesting that the olfactory system could be a model system in which to study the neurobiology of the disorder.

Concurrently, loss of ErbB4 leads to deficits in migration, placement and differentiation of MGE [9] and OB [10] interneurons.

The four ErbB4 isoforms - JMa-cyt1, JMb-cyt1, JMa-cyt2, JMb-cyt2 - originate from alternative splicing and are characterized by deep differences.

The presence of the exon "JMa" (in alternative to the exon JMb) confers to the receptor the ability to release the ectodomain into the extracellular environment and to release - through subsequent *regulated intramembrane proteolysis* [24] – the intracellular domain into the cytoplasm, where it can interact with other proteins and/or translocate into the nucleus.

The presence of the exon "cyt1" - which contains a tyrosine - confers to the receptor the ability to activate the PI3K and to interact with ubiquitin ligases, therefore becoming susceptible to degradation.

By preliminary qualitative assays we investigated the expression of the four ErbB4 alternatively spliced exons in the olfactory bulb system and we found that all of them were expressed (data not shown). To obtain quantitative data we analyzed by q-RT-PCR the expression of each exon and we found that all exons are transcribed. In particular, we observed that in the regions characterized by high migratory activity the expression level is higher; in the OB surrounding the RMS, where neuron migration stops, isoforms containing JMb exon are more expressed than those containing JMa, and cyt2 isoforms are more abundant than cyt1 ones, suggesting the existence of different roles played by different exons and isoforms. Actually, we previously showed *in vitro* that neural progenitor cells expressing JMa-cyt2 or JMb-cyt1 isoforms display higher NRG1 induced migratory activity

than cells expressing JMa-cyt1, and that neural progenitor cells expressing JMb-cyt2 have a very low migratory activity [14].

We hypothesized that the expression of JMb – which is not cleavable – would confer to the cells the ability to strongly adhere on a substrate of cells expressing transmembrane NRG1. On the contrary, the expression of the JMa exon, which is susceptible to proteolytic cleavage, would confer to the cells the ability to interact and then, following proteolytic cleavage, to go beyond. Accordingly to this hypothesis, JMb would mediate cell-cell attraction, JMa cell-cell repulsion, similarly to Eph receptors and ephrin ligands [25].

To investigate the cell-cell interactions mediated by the different ErbB4 isoforms, we examined how they behave when they interact with cells expressing transmembrane NRG1. To analyze the adhesion preference we performed the *Stripe Choice Assay*.

This assay is a useful method for studying binary growth decisions of cells or axons towards surface-bound molecules *in vitro* [26]. The method is based on the generation of a structured binary growth substrate, consisting of two sets of cues, arranged in alternating stripes, that can be generated with membrane fractions isolated from tissue or cells, with purified proteins or with cells expressing the candidate interacting proteins, usually belonging to a receptor/ligand pair.

The neural progenitor cell line chosen for this assay (ST14A) express ErbB1, ErbB2 and ErbB3; their expression could be a potential confounder, because ErbB2-ErbB3 is a known NRG1 receptor. Nevertheless, we previously demonstrated that wild type and mock cells do not migrate following soluble

NRG1 stimulation (Gambarotta et al., 2004) and here we demonstrate that the presence of ErbB3 and co-receptors ErbB1 and ErbB2 does not influence the preference for a cell substrate expressing transmembrane NRG1-type III: no statistically significant difference can be appreciated between cells adhering on NRG1 expressing cells and control cells.

Our data demonstrate that all ErbB4 isoforms confer to neural progenitor cells the ability to preferentially adhere on a cell substrate expressing transmembrane NRG1. However, no statistically significant differences among isoforms were observed.

The same results were obtained with the cell line COS7, transiently expressing ErbB4 isoforms, which lacks ErbB3. Moreover, we observed that the preference elicited by the different isoforms for NRG1 is in the same range of the preference elicited by MGE derived cells [9] and SVZ explants [10].

It is not known if transmembrane NRG1 is able of binding all isoforms with similar affinity. As far as we know, no papers were published describing the affinity of the different ErbB4 isoforms for NRG1. In a previous paper [14] we demonstrated that all isoforms can interact with soluble recombinant NRG1. Current data demonstrate, in an indirect way, that all ErbB4 isoforms interact (with unknown affinity) with the transmembrane NRG1 expressing clone, and that alternative splicing does not play a role in substrate preference.

Conclusions

Differences among the ErbB4 isoforms were expected, because of their deep difference. Actually, we hypothesized that JMa and JMb could play different roles

in cell-cell interactions. Nevertheless, the finding that all ErbB4 isoforms contribute in a similar manner to the adhesion preference elicited by the cells that express them, represents another step to the knowledge of the role played by ErbB4 and NRG1. Further analyses, *in vitro* and *in vivo*, could be carried out to learn more about ErbB4 and NRG1.

In vitro analyses using innovative proteomic strategies, such as the “quantitative analysis of Bidirectional Signaling” (qBidS), based on lineage-specific labeling with stable isotopomeric versions of amino acids (SILAC) [27], may allow to identify the signal transduction pathways activated downstream each ErbB4 isoform, both in the cell expressing the receptor (*forward signalling*) and in the cell expressing the ligand (*reverse signalling*).

In addition, *in vivo* electroporation of cDNAs coding for the different ErbB4 isoforms in the lateral ventricle may contribute to shed light on the role played by ErbB4 in neuronal migration.

Understanding the characteristics of the different ErbB4 isoforms will contribute to increase the knowledge of those phenomena in which ErbB4 isoforms are involved: neuronal migration, schizophrenia, breast cancer, heart development.

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FIGURE LEGENDS

Table 1 - Primers - Table containing forward and reverse primers used for quantitative real time PCR analysis. Ubiquitin C (UbC) and TATA box binding protein (TBP) were used as housekeeping genes to normalize data to the starting material.

Figure 1 - Structure of ErbB4 and its alternative isoforms. **A** - ErbB4 is a transmembrane tyrosine kinase receptor, containing at its N terminus two cysteine-rich domains (CRD), followed by a juxtamembrane domain (JM) which can be subject to alternative splicing (JMa/JMb), a transmembrane domain (TM), a tyrosine kinase domain (TK); a cytoplasmic domain (cyt) which can be subject to alternative splicing (cyt1/cyt2), a C terminus domain. **B** - Four ErbB4 isoforms are generated by alternative splicing of exons JMa (23 aminoacids), JMb (13 aminoacids), cyt1 (16 aminoacids): JMa-cyt1, JMb-cyt1, JMa-cyt2, JMb-cyt2 (cyt2 is the isoform lacking cyt1 exon). Isoforms containing the JMa exon can be cleaved by the metalloprotease TACE/ADAM17 (here represented by big scissors) to generate a soluble extracellular fragment, which contains the NRG1 binding site; the remaining membrane anchored receptor can be further cleaved by a γ -secretase preseniline-dependent protease (here represented by small scissors) that releases a cytoplasmic domain which can shift into the nucleus and interfere with transcriptional regulation. Isoforms containing the JMb exon are metalloprotease activity resistant.

Isoforms containing the cyt1 exon have an additional tyrosine residue which can interact with the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and with the

WW domain-containing E3 ubiquitin ligase (E3). The cyt2 isoforms, lacking cyt1 exon, do not interact with PI3K and are more resistant to ubiquitin mediated degradation.

Small arrows indicate the position of the primers used for q-RT-PCR. Forward primers for JMa and JMb are designed inside the JM exons, while the common reverse primer is in the adjacent downstream exon; reverse primer for cyt1 is inside the cyt1 exon, reverse primer for cyt2 overlaps the two exons flanking cyt1, the common forward primer is in the adjacent upstream exon. Primer sequence and amplicon size can be found in Table 1.

Figure 2 - A - *Schematic representation of a sagittal forebrain slice of postnatal day 5 (p5) mice.* (a) SVZ: the SVZ of the lateral ventricle; (b) pRMS: the posterior part of the RMS; (c) aRMS: the anterior part of the RMS; (d) sOB: the OB layers surrounding the aRMS. **B** - *Expression of the different ErbB4 isoforms in the olfactory bulb system* - The transcript copy number of the different ErbB4 exons JMa, JMb, cyt1 and cyt2 was quantified by absolute q-RT-PCR performed on microdissection samples obtained from p5 SVZ, pRMS, aRMS, sOB and the whole adult OB. Microdissections from six animals were pooled to obtain a suitable amount of RNA for one q-RT-PCR reaction, performed in technical triplicate; therefore, statistical analysis comparing all data was not feasible. When comparing all data relatively to microareas or isoforms, a significant difference between the aRMS microarea (in which all exons are highly transcribed) and the other areas is observed (see text). In the sOB region, containing differentiated interneurons, JMb isoforms seem more expressed than JMa.

Figure 3 - A - Cells stably expressing the transmembrane typeIII- β 3 isoform of the ligand NRG1 -Total protein extracts from mock cells and from a stable clone expressing NRG1 (N7) were separated on a 10% acrylamide gel and blotted on nitrocellulose membrane. The membrane was decorated with anti-NRG1 antibody directed to the N-terminus of the protein, that recognizes two specific bands of 83 and 44 kDa previously described [15] and studied [28]. The asterisk points to an unspecific band.

B - *Stripe choice assay method* - *Panel I* - ST14A cells stably expressing NRG1-typeIII- β 3 were plated in a 6 cm diameter plate, grown at approximately 95% confluence. *Panel II* - confluent cells were stained with Cell Tracker Green to obtain a green monolayer. *Panel III* - To produce the stripes, green cells were removed with a pipette tip (one line every 2 mm). *Panel IV* - mock cells were plated on top. Plates were incubated for 40 min to allow cell attachment to the empty stripes. The cell excess was washed out, then new medium was added. The same day, four ST14A stable clones, each expressing one of the four ErbB4 isoforms (and *mock* cells stably transfected with the empty vector) were transiently transfected with pDsRed1-N1 to obtain red fluorescent protein (RFP) expressing cells. (COS7 cells were co-transfected with the expression vector for one of the four ErbB4 isoforms and pDsRed1-N1). *Panel V* - 24 hrs later, cells in the stripes were grown to confluence. *Panel VI* - RFP-expressing cells were plated on top of cell stripes and incubated 24 hrs. For each plate, at least 15 stripes were photographed, paying attention to include in each photo both green

labeled and not colored stripes. In each photo, the green area, the not colored area and the number of red cells on green area and on not colored area were measured. For each sample, the number of red cells/green area and the number of red cells/not colored area were compared and expressed as percentages.

Representative images of cells displaying high (H) or low (L) substrate preference are shown. **C** - *Neural progenitor cells expressing different ErbB4 isoforms display adhesion preference for Neuregulin1-typeIII-β3 expressing cells.*

The adhesion preference elicited by different stable clones expressing the ErbB4 isoforms JMa-cyt1, JMb-cyt1, JMa-cyt2, JMb-cyt2 and mock cells was analyzed by stripe choice assay. For each ErbB4 isoform, two stable clones were analyzed in independent experiments (n=4 independent experiments, 15 green and 15 not colored fields examined for each experiment, *** p≤0,001). Control *mock* ST14A cells, although expressing the NRG1 receptor ErbB3-ErbB2, do not exhibit adhesion preference.

D - *COS7 cells expressing different ErbB4 isoforms display adhesion preference for Neuregulin1-typeIII-β3 expressing cells.* COS7 cells, transiently expressing different ErbB4 isoforms, were analyzed by *stripe choice* on NRG1-typeIII-β3-expressing ST14A cells (black bars) and control mock cells (white bars). (n=3 independent experiments, 15 green and 15 not colored fields examined for each experiment, *p≤0,05; **p≤0,01; ***p≤0,001). Control *mock* COS7 cells do not display adhesion preference.

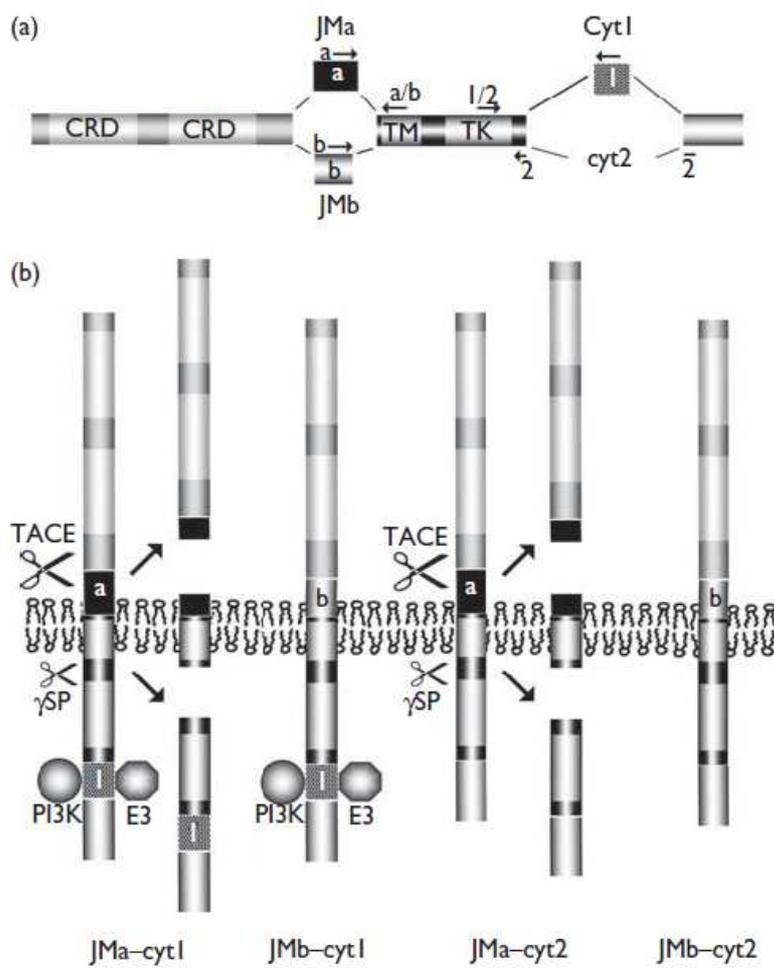


Figure 1

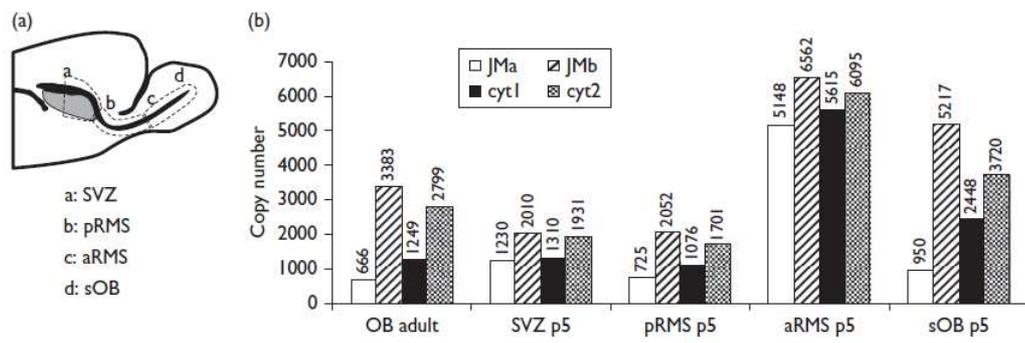


Figure 2

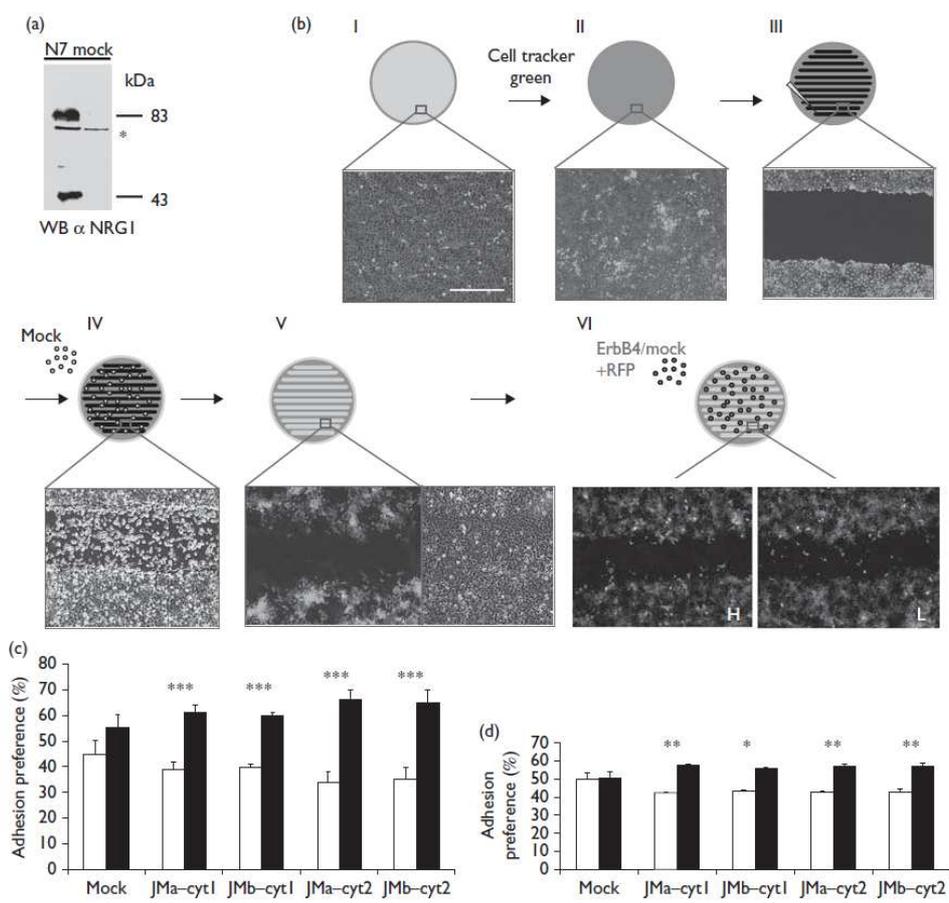


Figure 3

Table 1 Forward and reverse primers used for q-RT-PCR of *ErbB4* isoforms

	Forward	Reverse	Amplicon
ErbB4	mr_B4-JMa_FW (a)	mr_B4-JMa/b_REV (a/b)	67 bp
JMa	5'-GGACGGGCCATTCCACTTTACC-3'	5'-CCTCCAATGACTCCGGCTGC-3'	qRT-PCR
ErbB4	mr_B4-JMb_FW (b)	mr_B4-JMa/b_REV (a/b)	63 bp
JMb	5'-CATTGAAGACTGCATCGGCCTG-3'	5'-CCTCCAATGACTCCGGCTGC-3'	qRT-PCR
ErbB4	mr_B4-cyt1/2_FW (1/2)	mr_B4-cyt1_REV (1)	101 bp
cyt1	5'-GGAATATTTGGTCCCCCAGGCTTTC-3'	5'-GAGGAGGGCTGTGTCCAATTCAC-3'	qRT-PCR
ErbB4	mr_B4-cyt1/2_FW (1/2)	mr_B4-cyt2_REV (2)	91 bp
cyt2	5'-GGAATATTTGGTCCCCCAGGCTTTC-3'	5'-GTACACAACTGATTCCTATTGGAGTCAATTC-3'	qRT-PCR
TBP	m_TBP-FW	m_TBP-REV	106 bp
	5'-GATCAAACCCAGAATTGTTCTCC-3'	5'-GGGGTAGATGTTTCAAATGCTTC-3'	qRT-PCR
UbC	m_UbC-FW	m_UbC-REV	93 bp
	5'-CCACCAAGAAAGGTCAAACAGG-3'	5'-CCCATCACACCCAAGAACAAG-3'	qRT-PCR
ErbB4	mr_B4-JMa_FW (a)	mr_B4-cyt1_REV (1)	1248 bp
JMa-cyt1	5'-GGACGGGCCATTCCACTTTACC-3'	5'-GAGGAGGGCTGTGTCCAATTCAC-3'	
ErbB4	mr_B4-JMb_FW (b)	mr_B4-cyt1_REV (1)	1244 bp
JMb-cyt1	5'-CATTGAAGACTGCATCGGCCTG-3'	5'-GAGGAGGGCTGTGTCCAATTCAC-3'	
ErbB4	mr_B4-JMa_FW (a)	mr_B4-cyt2_REV (2)	1238 bp
JMa-cyt2	5'-GGACGGGCCATTCCACTTTACC-3'	5'-GTACACAACTGATTCCTATTGGAGTCAATTC-3'	
ErbB4	mr_B4-JMa_FW (a)	mr_B4-cyt2_REV (2)	1234 bp
JMa-cyt2	5'-GGACGGGCCATTCCACTTTACC-3'	5'-GTACACAACTGATTCCTATTGGAGTCAATTC-3'	

Ubiquitin C (UbC) and TATA box-binding protein (TBP) were used as housekeeping genes to normalize data to the starting material. The short name used in Fig. 1 is written in parenthesis. The amplicon size is given both for the amplification of the single exon [quantitative real-time PCR analysis (q-RT-PCR)] and for the amplification of the entire isoform (>1200 bp). Some primers can be used for both mouse (m) and rat (r) samples.

Table 1

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