Differential expression of neuregulins and their receptors in the olfactory bulb layers of the developing mouse.

Differential expression of neuregulins and their receptors in the olfactory bulb layers of the developing mouse

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ABSTRACT
Neuregulins (NRGs), and their cognate receptors (ErbBs), play essential roles in numerous aspects of neural development and adult synaptic plasticity. The goal of this study was to investigate the developmental expression profiles of these molecules during the olfactory bulb (OB) maturation. The OB is a highly organized structure with cell types and synaptic connections segregated into discrete anatomical layers. We employed a novel approach by combining single-layer microdissection at different development ages,with isoform-specific semi-quantitative RT-PCR and Western blotting to monitor layer-specific developmental profiles of these molecules and alternate splice variants. Layer and age specific regulation was observed for the ErbB4 splice variants JMa/JMb and NRG-1-β1/β2 forms. With the exception of the outermost (nerve) layer, ErbB4-JMb and NRG-1-β1 are expressed throughout the OB and their expressions decrease in the adult age in most layers. In contrast both ErbB4-JMa and NRG-1-β2 are highly expressed in the granule cell layer in the early postnatal OB. This early postnatal expression correlates with the dramatic change from radial glia to astrocytes and appearance of the bulk of granule cells occurring at this developmental stage.

Keywords: RT-PCR, Western blot, Radial glia, Neuronal development

Abbreviations:
EPL, external plexiform layer
GCL, granular cell layer
GL, glomerular layer
MCL, mitral cell layer
NRG, neuregulin
OB, olfactory bulb
ONL, olfactory nerve layer
RT-PCR, reverse transcriptase
polymerase chain reaction
TACE, tumor necrosis
factor-alpha-converting enzyme

1. Introduction

The neuregulins (NRGs) are a group of glycoproteins that belong to the epidermal growth factor (EGF)-like ligand family with widespread expression during development. At present, four genes encoding NRGs have been identified. The NRG-1–4 subfamily shares high sequence homology in their EGF-like domain, which is required for receptor binding and alternative splicing at the most carboxy-terminal region of the EGF-like domain gives rise to α and β isoforms (Wen et al., 1994). EGF domain β isoforms are prevalently expressed in the nervous system, whereas α isoforms are found in mesenchyme (Meyer and Birchmeier, 1994). Moreover, a region in this sub-domain, the

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linker, is also highly variable in both length and sequence and gives rise to different isoforms including β1 and β2 isoforms, of respectively intermediate and shorter length.
The neuregulins act through binding members of the EGF receptor (EGF-R) family. This EGF-R family consists of four members: ErbB1 (also called the epidermal growth factor receptor; EGFR), ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4 (Buonanno and Fischbach, 2001). ErbB proteins are transmembrane receptors with an extracellular-ligand-binding domain, a short transmembrane domain and an intracellular domain that has a tyrosine kinase catalytic activity, with the exception of ErbB3 which has a low functional kinase intracellular domain. ErbB4 has two alternative splice variants, JMa and JMb, which differ in the presence of a 23 or 13 amino acid sequences within the juxtamembrane region respectively. ErbB4-JMa is susceptible to a proteolytic cleavage (Vecchi and Carpenter, 1997) resulting in a fragment of approximately 85 kDa, representing the transmembrane and cytoplasmic domains of the molecule (Vecchi et al., 1996). This 85 kDa fragment can be processed by a second membrane-localized protease and translocated into the nucleus (Ni et al., 2001), where it associates to the transcriptional co-activators YAP-65 (Komuro et al., 2004; Omerovich et al., 2004) and STAT5A (Williams et al., 2004), suggesting possible involvement in transcriptional regulation. Although the EGF-like domains of neuregulins are similar, the binding specificities and affinities are different for the various combinations of ErbB receptors. For example, NRG-1 and NRG-2 bind to ErbB3 and ErbB4 (Carraway et al., 1997), whereas NRG-3 and NRG-4 bind preferentially to ErbB4 (Harari et al., 1999; Zhang et al., 1997). Following activation of the receptors with one of the several EGF family ligands, both homodimeric and heterodimeric combinations of receptors are induced, and their intrinsic catalytic tyrosine kinase activity stimulated (Yarden and Ullrich, 1998).
In the nervous system, NRG-1 activity has been implicated as a modulator of early fate determination, differentiation, migration and survival of glial cells (Buonanno and Fischbach, 2001). A crucial role for NRG-1 signaling has also been demonstrated in the cerebral cortex in establishment, maintenance and maturation of radial glial cells (Schmid et al., 2003). NRG-1 also specifically accelerates oligodendrocytes maturation and myelination, promoting ErbB4 cleavage and its nuclear localization (Lai and Feng, 2004). Neuronal–glia contact in the cerebellum induces morphological differentiation of radial glia via neuregulin-ErbB receptor signaling, and secondarily the ErbB receptor signaling is necessary for neuronal migration (Rio et al., 1997; Patten et al., 2003). Null mutations in ErbB3 result in severe abnormalities of midbrain and hindbrain development (Erickson et al., 1997), whereas ErbB4 deficient mice have abnormal targeting of cranial sensory and motor axons (Burden and Yarden, 1997). Taken together, these data suggest that NRG-1 is critical for the formation of glia in the brain.
The olfactory bulb (OB) is a well-organized structure with cell types and synaptic connections segregated into discernable anatomical layers (for review, Shipley et al., 2004). The OB undergoes considerable postnatal maturation with the bulk of interneurons generated postnatally (Bayer and Altman, 1975) and migrating into the bulb along the rostral migratory stream and through the granule cell layer (Lois and Alvarez-Buylla, 1994; Luskin, 1993). During the first postnatal week, there is also extensive reorganization of radial glia into astrocytes (Bailey et al., 1999; Puche and Shipley, 2001) and the formation of the unique glial tubes in the rostral migratory stream (Alves et al., 2002; Peretto et al., 2005). Anton et al. (2004) recently analyzed the expression of ErbB receptors and their ligands in the developing rat olfactory system. In situ hybridization showed that at postnatal day 11 (P11) ErbB4 is expressed at high levels in the rostral migratory stream and remains detectable in these cells as they migrate into the glomerular layer (GL) and granule cell layer (GCL) (Anton et al., 2004; Perroteau et al., 1998; Pollock et al., 1999). In adult, expression persists in granule neurons in the mature OB, but at reduced levels. In rat, ErbB3 expression at P11, and in adult at reduced levels, is prominent in ensheathing cells of the olfactory nerve layer but is almost absent in the internal layers (Anton et al., 2004; Perroteau et al., 1998; Pollock et al., 1999). However, little is known about the developmental expression profiles of the other ErbBs or any of the ErbB splice variants in mouse. Neuregulin-1 is expressed in the GCL, GL and in the mitral cell

layer (MCL); NRG-2 in the GCL, GL and in the external plexiform layer (EPL); NRG-3 in theMCLand GL of P11 and adult rat (Anton et al., 2004; Longart et al., 2004). Other reports have examined NRG 1–3 in the OB as part of expression pattern profiles in the entire mouse brain (Chen et al., 1994; Corfas et al., 1995; Longart et al., 2004; Meyer et al., 1997); however, comparative studies across the different OB layers and of the NRG splice variants during development have yet to be performed. Understanding the developmental expression patterns of the different splicing isoforms may contribute to understand their role in OB development.
Taking advantage of the laminar organization of the OB, we microdissected each layer and compared the developmental expression by parallel analysis of mRNA and protein from the same sample using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting. Unlike in situ hybridization, RT-PCR on single OB layers does not provide information on the expression at the cellular level, but allows detection and analysis, in one sample, of the relative abundance of several isoforms of the same molecule providing complementary information with respect to the cellular localization. We found a strong correlation between the temporal profiles of ErbB4 and NRG-1 in all layers, confirming our previous immunohistochemical findings in the adult deafferented OB (Oberto et al., 2001). In addition, we show that the NRG-1 isoform β2 and ErbB4-JMa, which are absent in the adult OB, are highly expressed in the prenatal granule cell layer. Other members of the NRG/ErbB family were also differentially expressed during development and across the bulb layers with less dramatic changes than NRG-1 and ErbB4.

2. Results

The olfactory bulb is a laminated structure with a highly organized distribution of different cell types to particular layers. Taking advantage of this laminar organization, we microdissected each layer and compared the expression of ErbB receptors and neuregulins by RT-PCR and immunoblotting. The olfactory nerve layer (ONL), glomerular layer (GL), mitral cell layer (MCL) and granule cell layer (GCL) were microdissected from E18, P0, P2, P4, P8, P16 and adult mouse olfactory bulbs. Due to size constraints, the external plexiform layer (EPL) could only be accurately microdissected from P8, P16 and adult animals. To correlate protein expression with mRNA expression, we employed the novel approach of extracting protein and RNA from the same sample. To test the accuracy of the microdissections, we probed the outer layers for the expression of olfactory maker protein (OMP) mRNA. OMP is a cytoplasmic protein whose expression is highly restricted to mature olfactory neurons (Margolis, 1980; Buiakova et al., 1994). In addition to protein, OMP mRNA is also distributed in the axons and nerve terminals (Wensley et al., 1995; Vassar et al., 1994; Ressler et al., 1994). Both OMP protein and mRNA are commonly accepted markers for the presence of mature ORN axons. OMP mRNA was observed in the ONL and GL layers; and only a faint signal was visible in the EPL (Fig. 1B) during development which is consistent with transient overshoot of ORN axons into the EPL (Bailey et al., 1999, Santacana et al., 1992; Tenne-Brown and Key, 1999). Moreover, as reported here and previously in the literature (Perroteau et al., 1998), no ErbB4 expression was detected in the ONL, whereas expression was present in the GL (Fig. 1I). These control localizations demonstrate precision in layer microdissections

2.1. Developmental and laminar-specific regulation of NRG and ErbB mRNA

The developmental/laminar expression profile of ErbB1, ErbB2, ErbB3, ErbB4, NRG-1, NRG-2, NRG-3 and splice variants was analyzed in microdissected layers by RT-PCR (Fig. 1). The expression of G3PDH was used as a control and was equivalent in all the layers and ages, suggesting an approximately equivalent amount of RNA in each sample (Fig. 1A). Splice variants were detected with primer pairs specific to the NRG-1-β1, α2 and β2 isoforms (Oberto et al., 2001), the ErbB4 juxtamembrane (JMa and JMb) and ErbB4 cytoplasmic (Cyt1 and Cyt2) isoforms. In the

case of ErbB4, only the Jma/b alternative splicing showed isoform-specific regulation, therefore only the JMa/b data are shown.
NRG-1-β1 and β2 isoforms are expressed in the OB with different developmental regulation (Fig. 1C), whereas NRG-1-α2 mRNA is almost undetectable. As shown in Fig. 2, the temporal expression

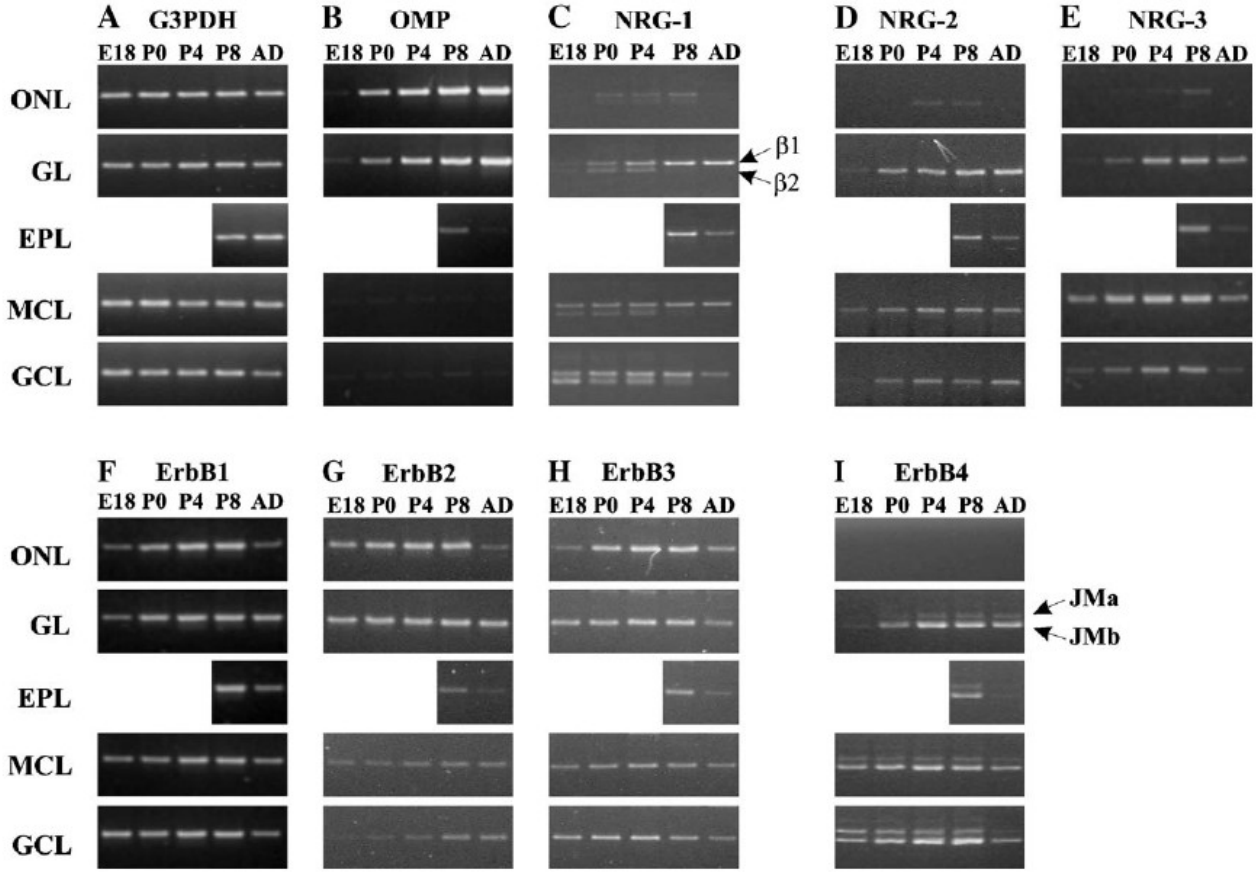

Fig. 1 – RT-PCR analysis of OMP, NRG-1 (β1 and β2 isoforms), NRG-2, NRG-3, ErbB1, ErbB2, ErbB3, ErbB4 (-JMa and -JMb) and G3PDH mRNAs across different olfactory bulb layers and at different ages. RNA was isolated from the olfactory nerve (ONL), glomerular (GL), external plexiform (EPL; P8 and adult only), mitral cell (MCL) and granule cell (GCL) layers of the developing mouse OB (E18, P0, P4, P8 and adult). RT-PCR amplifications products are 314 bp for OMP, 145 bp for NRG-1-β1, 120 bp for NRG-1-β2, 130 bp for NRG-1-α2, 240 bp for NRG-2, 299 bp for NRG-3, 377 bp for ErbB1, 270 bp for ErbB2, 237 bp for ErbB3, 246 bp for ErbB4-JMa, 207 bp for ErbB4-JMb and 173 bp for G3PDH as predicted. Representative gels selected from at least three independent experiments are shown

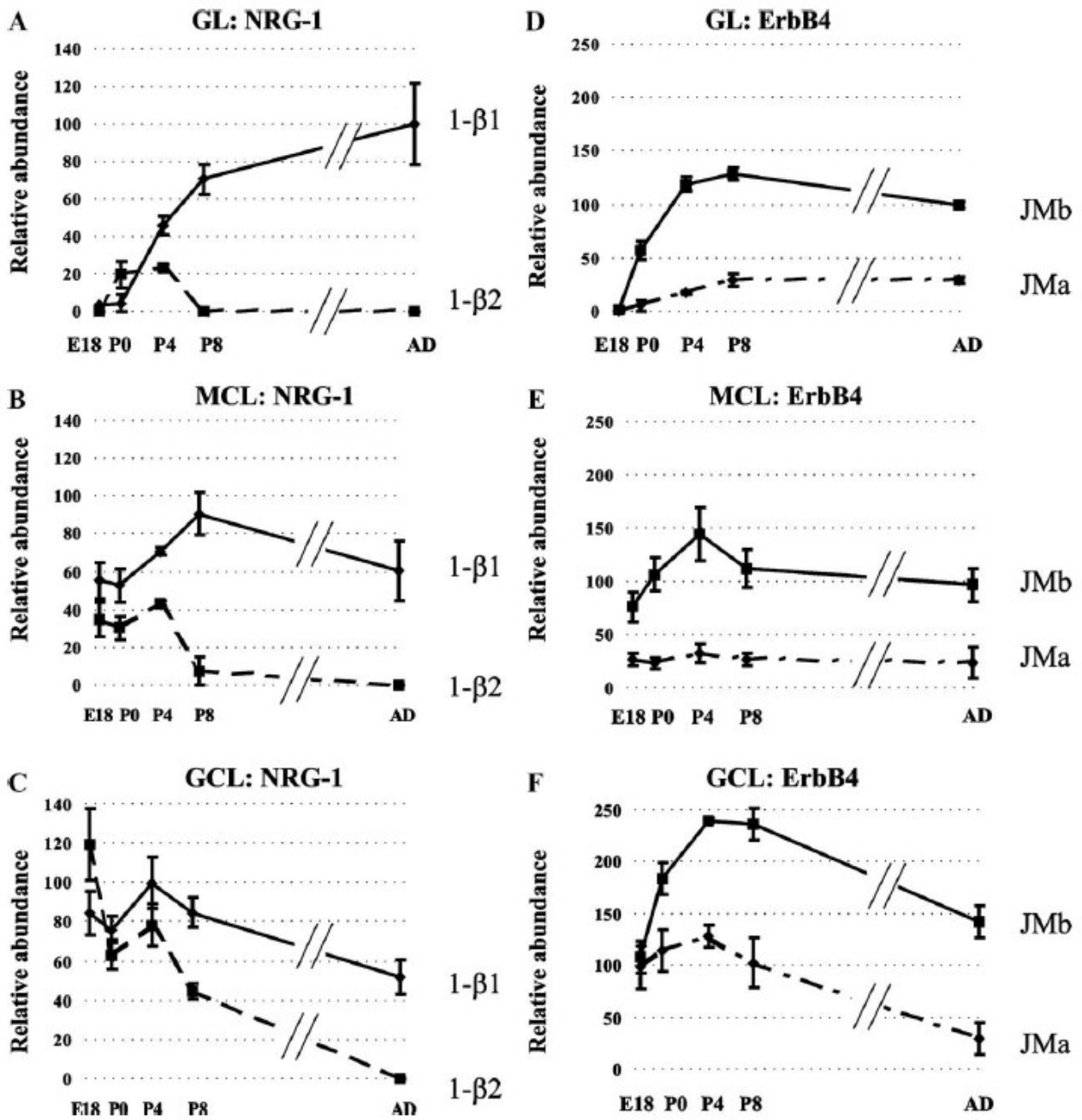

Fig. 2 – Semi-quantitative RT-PCR expression profiles of ErbB4-JMa/JMb isoforms and NRG-1-β1/β2 isoforms mRNA in the glomerular (GL), mitral cell (MCL) and granule cell (GCL) layers. Reactions were performed from at least three different reverse transcriptions, and results are shown as mean ± SEM of the independent RT-PCR reactions. Relative abundance is represented using the adult GL ErbB4-JMb and adult GL NRG-1-β1 as the reference (100%), all other layers/ages are shown as a percentage of this value.

of NRG-1-β1 and NRG-1-β2 vary in all layers (Figs. 2A–C). In the GL, NRG-1-β1 appears at P0 and increases through adult, while NRG-1-β2 is transiently expressed only between P0 and P4. In the MCL, NRG-1-β1 is expressed at E18 showing peak expression at P8, while the NRG-1-β2 isoform is transiently expressed only during the first postnatal week. In the GCL, NRG-1-β1 is constitutively expressed at high levels with slightly decrease through adult. NRG-1-β2 expression is greater than β1 before birth, but decreases through postnatal development and is absent in adult. NRG-2 and NRG-3 mRNAs (Figs. 1D–E) show similar laminar distribution in the bulb, but different developmental trends. Both NRG-2 and NRG-3 mRNAs increase at birth in the GL and

GCL, but only NRG-3 shows a decline into adult, especially in the GCL.
Like many NRGs, ErbB receptor mRNAs levels are higher during the first postnatal week followed by expression decline in the adult (Figs. 1F–I). ErbB1, ErbB2 and ErbB3 show a similar expression pattern in the ONL while ErbB4 is absent. The expression trend of ErbB1 and ErbB3 mRNAs is also similar in the MCL and GCL, where their expression is elevated until P8 and decreases in the adult. The ErbB4-JMb mRNA is more abundant than ErbB4-JMa in all layers and ages examined, except in the GCL at E18 where the two isoforms are equally expressed. The ErbB4 distribution and the developmental profile of the JMb isoform most closely resemble that of NRG-1-β1, with the only difference that the latter follows with a slight developmental delay (see shift in mRNA increase in Jmb at E18 and NRG-1-β1 at P0; Fig. 2). The ErbB4-JMa mRNA is generally very low except at early developmental stages in the GCL (Fig. 2F).

2.2. Laminar distribution of ErbB proteins in the adult olfactory bulb

To analyze the distribution of ErbB proteins in the adult olfactory bulb, we examined protein expression in individual adult olfactory bulb layers (Fig. 3). ErbB1 protein expression is similar to the mRNA distribution, with the strongest expression in the GL and reduced levels in the deeper layers (Fig. 3A). Expression in the ONL is very low. ErbB2 is primarily present in the outer bulb layers with the strongest ErbB2 expression in the ONL and GL and weak signal detected in the EPL and MCL (Fig. 3B). Previous immunocytochemical work in our laboratory showed the expression of ErbB3 in ensheathing cells localized in the ONL and the outer GL and the expression of ErbB4 in periglomerular cells (GL), mitral/tufted cells (EPL and MCL) and in a small number of cells in the GCL (Perroteau et al., 1998; Oberto et al., 2001). Consistent with these data, here, we report ErbB3 protein expression only in the ONL and GL (Fig. 3C) and ErbB4 protein expression in all bulb layers except the ONL (Fig. 3D). As described in Experimental procedures, the immunoblotting analysis using an ErbB4 antibody, performed on ST14A cells that do not express any ErbBs, shows that the two bands observed at 60 and 80 kDa are non-specific.

2.3. Developmental regulation of ErbB4-JMa and Jmb splice variants in the olfactory bulb

The ErbB4 antibody used in this study recognizes the fulllength ErbB4-JMa and ErbB4-JMb proteins as well as the 85 kDa proteolytic cleavage product of ErbB4-JMa. In ST14A cells stably transfected with the full-length ErbB4-JMa, it shows a 180 kDa full-length ErbB4 and a ∼85 kDa band (Fig. 4A), likely corresponding to the cytoplasmic fragment derived from the cleavage of ErbB4-JMa by tumor necrosis factor-alpha-converting enzyme (TACE; Rio et al., 2000). ST14A cells stably transfected with ErbB4-JMb or vector alone did not show this 85 kDa band.
Western blot analysis using the same ErbB4 antibody performed on protein extracts from developing whole OB (30 μg total protein per lane) also detected the 180 kDa full-length product, two unspecific ∼60 and ∼80 kDa bands and the 85 kDa band corresponding to the JMa cleavage fragment (Fig. 4B). The 85 kDa fragment was abundant at postnatal day 0 and declined rapidly at P8 through adult (Fig. 4B). These results suggest that the cleavage of ErbB4-JMa in the olfactory bulb occurs mainly at early developmental ages.

2.4. Comparison of ErbB3 and ErbB4 proteins and mRNA expression

Parallel extraction of mRNA and proteins from the same sample permits a direct comparison of the relative mRNA expression levels with the protein levels. Our data show that both ErbB4 mRNA and protein are absent from the ONL and
present in the GL starting from P0 (Figs. 5A–B). The expression of mRNA correlates to protein expression throughout most layers and ages. However, in the GL, the mRNA level remains elevated from P4 throughout adult, whereas the protein levels are dramatically lower in the adult. The 85

kDa band observed in OB whole extracts (Fig. 4) was not detected in these layer samples, most

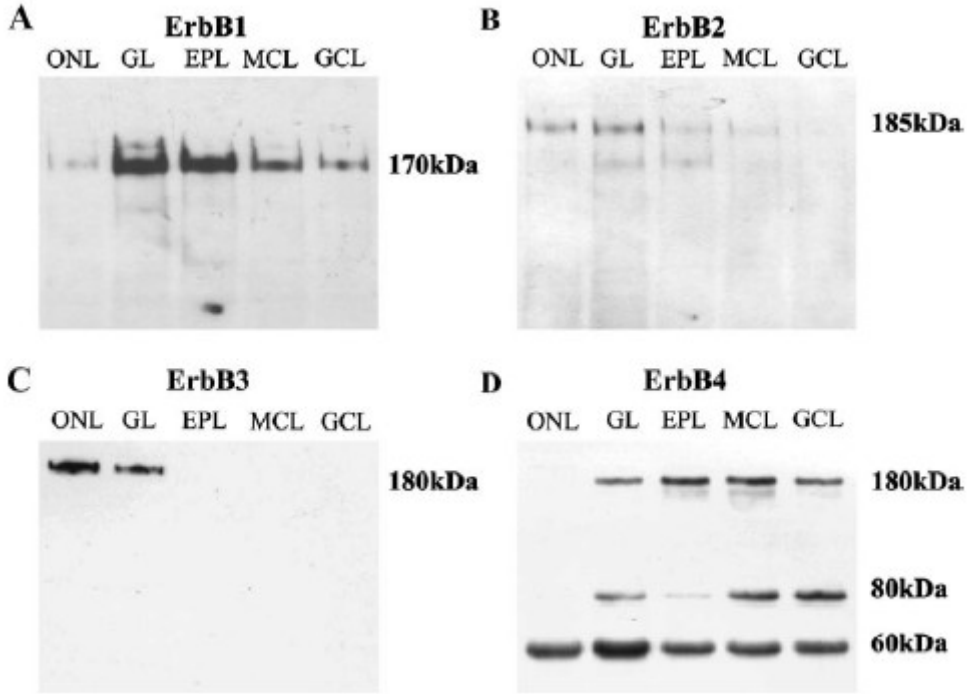

Fig. 3 – Expression of ErbB protein in the adult olfactory bulb. Equal amounts of proteins (30 µg/lane) extracted from microdissected layers of the mouse olfactory bulb (ONL: olfactory nerve; GL: glomerular; EPL: external plexiform; MCL: mitral cell; and GCL: granular cell layers) were run on polyacrylamide gels, blotted and incubated with ErbB1, ErbB2, ErbB3 and ErbB4 antisera. Representative immunoblots of at least three independent experiments are shown.

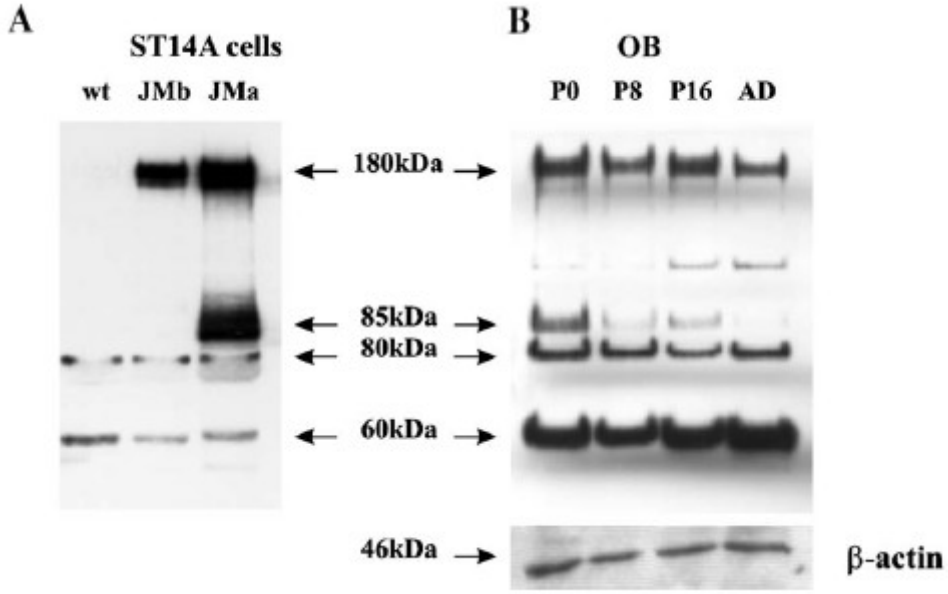

Fig. 4 – Expression of ErbB4 protein splice variants in ST14A cell line (A) and developing whole olfactory bulb (B). (A) Protein extracts from the ST14A cell line were used as a control to test specificity of the ErbB4 antibody. Lane 1, untransfected ST14A cells; lane 2, transfection with ErbB4-JMa; and lane 3, transfection with ErbB4-JMb. In control non-transfected ST14A cells, two non-specific bands at 60 and 80 kDa are present. Transfection with ErbB4-JMb results in a single specific band at the expected 180 kDa full-length ErbB4 protein, whereas transfection with ErbB4-JMa results in a 180 kDa full-length ErbB4 product and an 85 kDa product consistent with the predicted proteolytic cleavage fragment of the JMa protein. (B) In whole olfactory bulb protein extracts, the ErbB4 antibody also recognized the non-specific 60 and 80 kDa bands. The expected 180 kDa full-length ErbB4 was present at all ages, with a slight reduction in expression levels from P0 through adult. The 85 kDa ErbB4-JMa cleavage fragment was prominently expressed at P0, trace amounts were detectable at P8 and P16 but were undetectable by adult.

likely due to the very low abundance of the cleavage product and the small sample sizes of this layer material.

In contrast to ErbB4, ErbB3 is abundant in both ONL and GL. The expression is strongest in the postnatal period with sharp decline in the adult (Figs. 5C–D). Comparable to ErbB4, the mRNA expression level for ErbB3 in the adult GL is similar to the postnatal GL; however, the protein level in adult dramatically declines. This observation suggests a strong translational control of ErbB3 as well as ErbB4 in adult GL. Interestingly, using the method of parallel mRNA and protein extraction, weak bands corresponding to ErbB3 mRNA and protein are present in both the MCL and GCL from P4 to adult.

3. Discussion

Neuregulins (NRGs) and their receptors, the ErbBs, play important roles in the development of the central nervous system by regulating both neuronal and glial precursor proliferation, migration, differentiation and survival (Burden and Yarden, 1997; Gassmann and Lemke, 1997; Lemke, 1996; Longart et al., 2004). Understanding the functional roles of these molecules in the nervous system is complicated by the existence of numerous splicing variants whose specific temporal and spatial expression profiles are still poorly characterized. The present work specifically addresses this issue by analyzing the developmental expression of several NRGs and ErbBs splicing forms in the olfactory bulb and correlating expression with the invariant sequence of developmental events that lead to amature olfactory system (Bailey et al., 1999; Treloar et al., 1999; Puche and Shipley, 2001). The olfactory bulb is one of the first places in the brain to undergo astrocyte differentiation in late embryogenesis, with the bulk of olfactory astrocytes appearing to transform from radial glial cells from E18 to P8 (Puche and Shipley, 2001). The first mature astrocytes are observed first in the glomerular layer and later in the deep layers of the bulb. However, during transdifferentiation of radial glia to astrocytes, radial glia cell bodies translocate from the deeper bulb layers to the GL and begin expressing GFAP in intermediate ‘radial astrocytes’. At the same time, olfactory bulb radial glia form specialized apical tufts and side branches at E17–18 that are hypothesized to help form the cytoarchitecture of glomeruli and bulb lamination (Puche and Shipley, 2001). Morphological differentiation of radial glia in the cerebellum is influenced by neuregulin-ErbB receptor signaling via NRG-1 and ErbB4 (Rio et al., 1997; Patten et al., 2003). NRG-1 and ErbB4 are expressed in the olfactory bulb during the time radial glia undergo structural reorganization and differentiation into astrocytes. In the bulb, the NRG-1-β2 isoform is selectively expressed between E18 and P4 and the β1 isoform from P0 to adult. These expression patterns correlate with glial differentiation and/or maintenance in the bulb. We hypothesize that, as in the cerebellum (Rio et al., 1997; Patten et al., 2003), the NRG-1/ErbB4 are important for glial differentiation.

ErbB4 is present in two alternate splice forms, JMa and Jmb, which differ by the presence of either a 13 or 23 amino acid insert. The JMa splice form can be cleaved yielding a 120 kDa extracellular soluble fragment and an 85 kDa fragment (Vecchi et al., 1996). This 85 kDa fragment can translocate into the nucleus (Ni et al., 2001), where it could be involved in transcriptional regulation (Williams et al., 2004; Komuro et al., 2004; Omerovich et al., 2004). Although little is known about the role of this fragment into the nucleus, it has been recently demonstrated that it has an important role in regulating oligodendrocytes maturation induced by NRG (Lai and Feng, 2004). Our data show the presence of this fragment only in early postnatal bulb development when oligodendrocytes are maturing (Jacque et al., 1985), consistent with the proposed functional role of the cleavage product (Lai and Feng, 2004).

Ozaki and colleagues analyzed the expression of ErbB2, ErbB3 and ErbB4 proteins and mRNA in the developing mouse cerebellum, showing strong expression of the receptors in the postnatal period with a peak at P18 followed by a strong decrease in the adult age (Ozaki et al., 1998). This matches the peak of major synaptic organization of the cerebellum and is consistent with roles for

neuregulin in synaptogenesis (Ozaki et al., 2000). In the olfactory bulb, ErbB3 and ErbB4-JMb reach maximal expression around P4–P8 and decrease toward the adult age. Interestingly, the

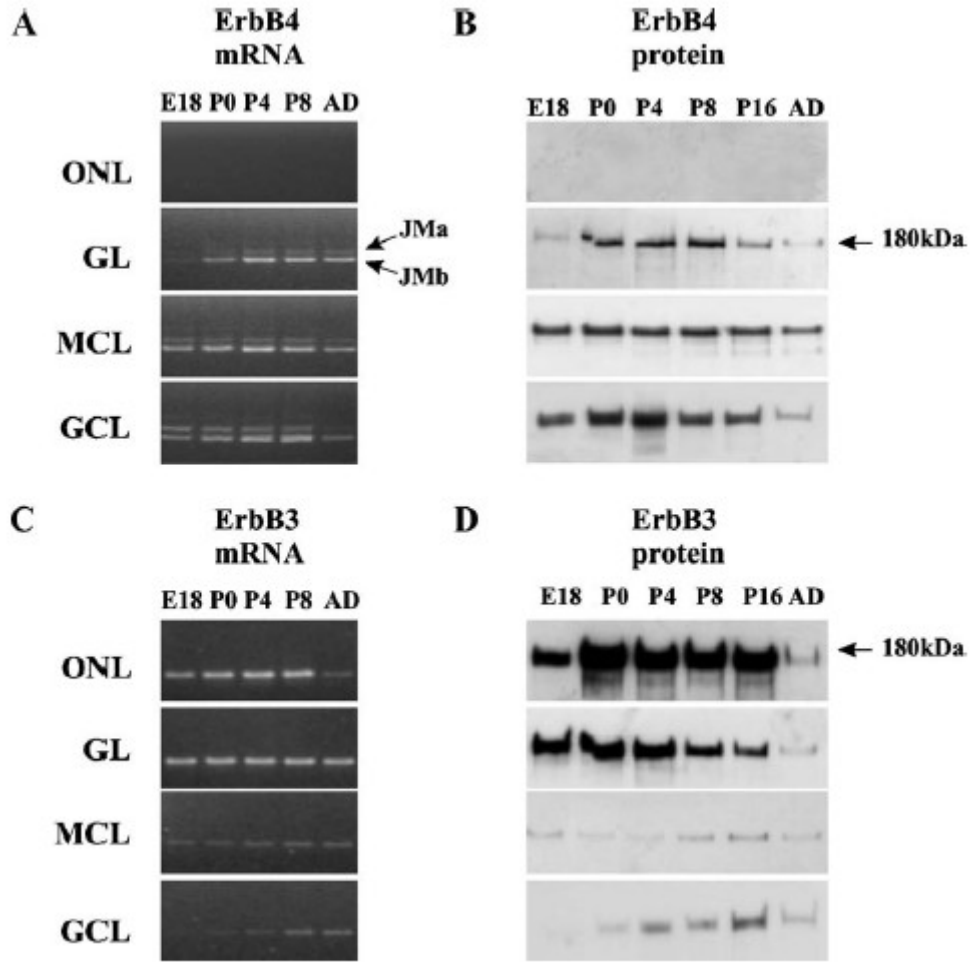

Fig. 5 – Developmental profile of ErbB4 and ErbB3 mRNA and protein expression in different layers of the developmental mouse OB. (A) ErbB4 mRNA expression analysis by RT-PCR. ErbB4 was expressed in the glomerular (GL), mitral cell (MCL) and granule cell (GCL) layers but was absent from the olfactory nerve layer (ONL). Expression of the ErbB4-JMa splice variant declined during development to barely detectable levels in adult. ErbB4-JMb expression persisted into adult, with the strongest expression maintained in the GL. (B) Protein expression analysis by immunoblotting. Expression of the ErbB4 protein paralleled mRNA expression levels except in the adult glomerular layer. Data are representative of at least two independent experiments. (C) ErbB3 mRNA expression analysis by RT-PCR. ErbB3 was expressed in the ONL and GL during development. In the adult, expression in the ONL was reduced. In the deeper bulb layers, MCL and GCL, ErbB3 was only weakly detectable. (B) Protein expression analysis by immunoblotting. ErbB3 protein is abundantly expressed in the outer bulb layers (ONL and GL) but downregulates in the adult. Despite persistent expression of mRNA for ErbB3 in the GL, the protein downregulates in the adult GL, suggesting a translational control in the adult GL. Low levels of ErbB3 protein were detectable in the deeper bulb layers consistent with the expression of mRNA in these layers. Data are representative of at least two independent experiments.

majority of interneurons, periglomerular and granule cells, are born and migrate into the bulb during this early postnatal period (Bayer and Altman, 1975) and could require neuregulins/ErbBs to establish appropriate synaptic connections.

In adult, previous immunocytochemical work in our laboratory showed the expression of ErbB3 in the ensheathing cells of the olfactory nerve and the outer GL, whereas ErbB4 immunoreactivity was found in periglomerular interneurons (GL), in mitral/tufted cells (EPL and MCL) and few cells of the granule cells layer (Oberto et al., 2001; Perroteau et al., 1998). Consistent with these data, we found that ErbB3 transcripts and proteins are prominently expressed in the external layers of the OB

(ONL and GL). Interestingly, using an extraction buffer (standard HES) which preferentially isolates the membrane fraction from the tissue, ErbB3 protein is completely absent from all layers except the ONL and GL. Surprisingly, the parallel extraction of mRNA and protein by using TRIzol, which extracts all proteins (cytoplasmic, membrane and nuclear), shows the presence of ErbB3 protein at low levels also in the MCL and GL. This observation suggests that in late postnatal and adult ErbB3 mRNA in the MCL and GCL is translated into protein, but a very small portion of it is incorporated into cell membrane. Insertion of a small fraction of the protein, with a cytoplasmic reserve pool, could be a mechanism to regulate ErbB function in the adult.

4. Experimental procedures

4.1. Animals

CD-1 adult mice (25–30 g body weight) and neonates (Charles River) were maintained under a 12-h light, 12-h dark cycle. Animals were anesthetized by 100 mg/kg sodium pentobarbital for all procedures. Animal care was in accordance with D.L. 116/92 from the Italian Government and received the approval of the animal ethics committee of the University of Turin and of the University of Maryland Institutional Animal Care Usage Committee (IACUC).

4.2. Isolation of olfactory bulb layers

Microdissections of individual olfactory bulb layers were performed on fresh 300 μm vibratome slices harvested from the olfactory bulbs of E18, P0, P2, P4, P8, P16 and adult mice, under a high magnification-dissecting microscope. The layers microdissected were: the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL) and granule cell layer (GCL). Microdissection approximately 25 μm superficial to the GL separated the ONL, while dissection approximately 25 μm deep to the GL separated this layer. Dissection approximately 25 μm either side of the MCL separated the EPL, MCL and GCL. Therefore, the ONL and EPL samples do not contain any material from the GL, whereas the GL layer contains small amounts of ONL and EPL. Furthermore, the EPL and GCL samples do not contain any MCL material, but the MCL contains small amounts of EPL and GCL. Each layer was microdissected from multiple animals at each age (6 adult animals, 4 animals at P16; 6 animals at P8; and 10 animals at each of E18, P0, P2 and P4). Each layer was frozen on dry ice and stored at −80 °C until used for RNA and protein extraction.

4.3. RNA/protein isolation

Total RNA and proteins were extracted from each sample with TRIzol (Invitrogen™ Life-Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions with the following modifications. In brief, each sample was homogenized in 800 μl of TRIzol by passing through a series of 18–23 g needles. To facilitate the precipitation of total RNA from small samples, 250 μg/ml of glycogen (Ambion, Austin, TX) was added as a carrier with the TRIzol reagent. Following the addition of chloroform and centrifugation, the upper (RNA containing) aqueous phase was transferred to a fresh tube and RNA precipitated by isopropyl alcohol. The RNA pellet was resuspended in sterile water and stored at −80 °C until use. After removal of the aqueous phase, DNA was precipitated from the interphase with ethanol. Total tissue proteins were precipitated from the remaining organic phase with isopropyl alcohol. The protein pellets were washed 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol, vacuum-dried, resuspended in SDS 1% and stored at −20 °C until use. This protocol yielded both RNA and proteins from the same sample.

4.4. RT-PCR

RNA yield was quantified by measuring absorbance at 260/280 nm. Total RNA (500 ng from each layer) was reversetranscribed to cDNA in a reaction volume of 20 µl, containing 200 U of Superscript II Reverse Transcriptase (Invitrogen™ Life-Technologies, Inc., Grand Island, NY), 2.5 µM random hexamers (Amersham-Pharmacia Biotech, Piscataway, NJ), 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 2 U/µl RNAse inhibitor (Amersham), 10 mM DTT, 1 mM deoxynucleotide triphosphates (dNTPs, Amersham). RNA was denatured for 5 min at 65 °C and incubated in the reaction mixture for 10 min at room temperature followed by 50 min at 42 °C and 15 min at 70 °C.

PCR reactions were carried out in a total volume of 30 µl including 3 µl of cDNA, 0.5 µM each of specific 5′ and 3′ primers, 1× standard reaction buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin) (Sigma-Aldrich, St. Louis, MO), 200 µM dNTPs (Amersham) and 0.025 U/µl of REDTaq DNA polymerase (Sigma). Specific primers designed to amplify G3PDH, OMP, ErbB1, ErbB2, ErbB3, ErbB4 (CYT1/2), ErbB4 (JM a/b), NRG-1 (β1/α2/β2), NRG-2 and NRG-3 are listed in Table 1. G3PDH amplification (27 cycles, 66 °C) was used to monitor the quality and quantity of RNA that had been reverse-transcribed into cDNA. Samples were amplified for 32–35 cycles, depending on the primer pair (Table 1). Cycling parameters were set for each primer pair in order to obtain specific products in the exponential amplification phase of the PCR reaction. Specificity of the primer pairs was verified by sequencing the PCR product. Amplification products were separated by agarose gel electrophoresis and DNA bands visualized with ethidium bromide staining. Negative controls were performed from reverse transcription reactions lacking either reverse transcriptase or total RNA.

For quantitative PCR, cDNA was diluted 1:4 in water and, for each sample, series of cDNA aliquots (2, 4, 6 µl) were amplified with RedTaq polymerase using specific primer pairs. After electrophoretic separation and digital image acquisition of ethidium-bromide-stained gels (Gel Doc, Bio-Rad), the intensity profile of individual bands was measured using the Phoretix gel analysis software (Nonlinear Inc, Durham, NC). Integrated optical intensity of each band was plotted against cDNA volume, and the abundance of each gene was calculated in the exponential phase of the amplification from values obtained by linear regression analysis (Giustetto et al., 1997). Results from experiments from at least three different cDNAs were averaged and expressed as mean ± standard error of the mean (SEM).

Table 1 – PCR amplification primers

Gene	Primer sequence	Accession number, species, product size
G3PDH	5′-TTTGCCGTGAGTGGAGTCATACTG-3′ 5′-CGTCCCGTAGACAAAATGGTGAAG-3′	M32599, mouse, 173 bp
OMP	5′-GGGAGAAGAAGCAGGATGGTGAGA-3′ 5′-ATACATGACCTTGCGGATCTTGGC-3′	U01213, mouse U02557, mouse, 314 bp
ErbB1	5′-AGCAAGGCTTCTTCAACAGC-3′ 5′-AGACAGGTAGGCTGGGCAGT-3′	AF275367, mouse, 377 bp
ErbB2	5′-GCTGGTCGATGCTGAAGAGT-3′ 5′-GAGGTCATGTGGAGAGAGGC-3′	L47239, mouse, 270 bp
ErbB3	5′-GGAGGCTTGTCTGGATTCT-3′ 5′-GGGAGTAAGCAGGCTGTGT-3′	L47240, mouse, 237 bp
ErbB4 (cyt1/2) [a]	5′-TGCTGAGGAATATTTGGTCCCCCA-3′ 5′-TCTGGTATGGTGCTGGTTGTGGCT-3′	AF059177, mouse, 214 bp (cyt1) L47241, mouse, 167 bp (cyt2)
ErbB4 (JMa/b) [b]	5′-GAAATGTCCAGATGGCCTACAG-3′ 5′-AACGGCAAATGTCAGAGCCATG-3′	NM_021687, rat, 246 bp (JMa) and 207 bp (JMb)
NRG-1 (β1/α2/β2) [a]	5′-TGAAAGACCTTTCAAACCCCTC-3′ 5′-CTCTTCTGGTACAGCTCCTCCG-3′	NM_013964, human, 120 bp (β2), 130 bp (α2), and 145 bp (β1)
NRG-2 [a]	5′-GAAGATGAAGAGCCAGACGG-3′ 5′-GTCCTTCCCCAGGATGTTCT-3′	NM_004883, human, 240 bp
NRG-3	5′-GACAGGATCCCATAAGCACTGT-3′ 5′-GATGCCTTGAGGCTGTAGTTCT-3′	AF010130, mouse, 299 bp

[a] Primer sequences from reference Ozaki et al., 1998.
[b] Primer sequences from reference Gerecke et al., 2001.

4.5. Western blotting analysis

Proteins from single OB layers, the entire OB, ST14A cell line and two ST14A cell line clones expressing the ErbB4 isoforms JMb-cyt2 and JMa-cyt2, were extracted with TRIzol (Invitrogen ™ Life-Technologies, Inc., Grand Island, NY) as described above. In a separate set of experiments, proteins were extracted from single layers of adult mouse OB, using HES (20 Mm HEPES pH 7.4, 1mMEDTA, 250mMsucrose) (Gerecke et al., 2001). The protein content of whole OB and the ST14A cell lines was determined using a bicinchoninic acid kit for protein determination (Sigma). Due to small amounts of protein present in a single OB layer, a protein spot density method (Bannur et al., 1999; Mautino et al., 2004) was used to quantify proteins extracts from single layers of the developing mouse OB. Briefly, a constant volume (1 μl) of protein solutions was spotted on nitrocellulose paper, stained with acid Ponceau S (Ponceau S 0.1%, acetic acid 5%), destained and air-dried. The integrated density of each spot was measured using Total Lab analysis Program (Phoretix) and compared to protein standards similarly spotted.

Western gels were prepared using 10 μg or 30 μg of proteins released by boiling in Laemmli loading buffer (2% SDS, 50 mM Tris–HCl pH 7.4, 20% β-mercaptoethanol, 20% glycerol) and analyzed by 8% SDS polyacrylamide gel electrophoresis (PAGE). Proteins were blotted onto Hybond membrane (Amersham) according to the manufacturer's instructions. After blocking with 5% nonfat powder milk in TBST buffer (20 mM Tris, 150 mMNaCl, 0.1% Tween-20, pH 7.4), filters were probed with antibodies diluted 1:500 in TBST containing 1% nonfat powder milk. Proteins were visualized with the appropriate peroxidase-coupled secondary antibodies by using the enhanced chemiluminescence (ECL) detection system (Amersham).

Polyclonal antibodies against ErbB1 (Santa Cruz), ErbB2 (Santa Cruz), ErbB3 (Santa Cruz), ErbB4 (Santa Cruz) and β-actin (Sigma) were used for staining Western blots. Standardization of protein loading was performed both by total protein measurements (described above) and comparison of the β-actin band intensity. Each membrane was sequentially probed with 2 antibodies. After the first antibody incubation and detection, membranes were incubated at 60 °C for 30 min in a stripping solution containing 0.05 M sodium phosphate, pH 6.5; 10 M urea; 0.1 M 2-mercaptoethanol following the Hybond membrane instructions. The efficiency of the stripping procedure was assessed by incubating the membrane with an appropriate peroxidase-coupled secondary antibodies and confirming the absence of signal using the ECL detection system. Only membranes that were completely free of signals were used for incubations with a second primary antibody. All samples were tested on at least two different separations, Western blots and immunoreactions, a representative blot is shown in each figure.

Controls included: (1) membrane incubation with antibodies preabsorbed with a correspondent blocking peptide (ErbB1, ErbB2, ErbB3 and ErbB4; Santa Cruz); (2) immunoblotting of wild type and transfected ST14A cells. Two stable clones exogenously expressing the ErbB4 isoforms, Jmb-cyt2 and JMa-cyt2, were used as positive controls for ErbB4 expression. These cells were obtained by stable transfection of a cell line (ST14A) (Cattaneo and Conti, 1998) with the vector pIRES-puro2 (Clontech) expressing rat ErbB4 cDNAs (Gambarotta et al., 2004) corresponding to all known different isoforms (Kainulainen et al., 2000; Elenius et al., 1997). The wild type cell line (ST14A), which does not express ErbB4, was used as a negative control.

All antisera used for these studies produced an immunoreactive band of the predicted molecular weight for the fulllength receptor (ErbB1 ∼170 kDa, ErbB2 ∼185 kDa, ErbB3 ∼180 kDa, ErbB4 ∼180 kDa; Fig. 4), and all immunoreactive bands disappeared with antibody preabsorption with the corresponding synthetic peptide antigen (data not shown). In addition to the expected 180 kDa band, the ErbB4 antibodies labeled two additional bands: a 60 kDa band, present in all layers, and

an 80 kDa band in the GL, MCL, and GCL. These 60 kDa and 80 kDa were present in the ST14A cell line lacking ErbB4 expression, whereas the expected 180 kDa band was absent in this cell line. Thus, we conclude the 80 kDa and 60 kDa bands are non-specific cross-reactivity of this antibody.

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