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Neuregulin1 alpha activates migration of neuronal progenitors expressing ErbB4

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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.

1. 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

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, tumour necrosis factor-α-converting enzyme; TBP, TATA box Binding Protein.

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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.

2. Material and methods

2.1. 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.

2.2. 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.

2.3. 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: 1× 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 15 ng starting RNA) were analysed in a 20 μl reaction volume, containing 1× 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).

2.4. 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 200 μl 2% FBS DMEM were seeded in the upper chamber of the cell culture insert, on a porous transparent polyethylene terephthalate membrane (8.0 μm pore size, 1×10^5 pores/cm^2). The lower chambers contained 800 μl 2% FBS DMEM as control condition, or 2% FBS medium added with NRG1α (200 ng/ml, 28.5 nM) or NRG1β (50 ng/ml, 6.24 nM). Cells were allowed to migrate 18 h 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 min at room temperature, washed with water and then stained with 0.1% crystal violet in 20% methanol. Transwells were photographed (8-bit images, 4× magnification) using a Nikon ECLIPSE TS100 inverted microscope equipped with a