Experimental Neurology 302 (2018) 85–92

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

An essential role for neuregulin-4 in the growth and elaboration of developing neocortical pyramidal dendrites

Blanca Paramo, Sean Wyatt, Alun M. Davies*

School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, Wales, UK

ARTICLE INFO

Keywords:
Neuregulin
Cortical pyramidal neuron
Dendrite
Knockout mouse
Development

ABSTRACT

Neuregulins, with the exception of neuregulin-4 (NRG4), have been shown to be extensively involved in many aspects of neural development and function and are implicated in several neurological disorders, including schizophrenia, depression and bipolar disorder. Here we provide the first evidence that NRG4 has a crucial function in the developing brain. We show that both the apical and basal dendrites of neocortical pyramidal neurons are markedly stunted in *Nrg4*$^{-/-}$ neonates *in vivo* compared with *Nrg4*$^{+/+}$ littermates. Neocortical pyramidal neurons cultured from *Nrg4*$^{-/-}$ embryos had significantly shorter and less branched neurites than those cultured from *Nrg4*$^{+/+}$ littermates. Recombinant NRG4 rescued the stunted phenotype of embryonic neocortical pyramidal neurons cultured from *Nrg4*$^{-/-}$ mice. The majority of cultured wild type embryonic cortical pyramidal neurons co-expressed NRG4 and its receptor ErbB4. The difference between neocortical pyramidal dendrites of *Nrg4*$^{-/-}$ and *Nrg4*$^{+/+}$ mice was less pronounced, though still significant, in juvenile mice. However, by adult stages, the pyramidal dendrite arbors of *Nrg4*$^{-/-}$ and *Nrg4*$^{+/+}$ mice were similar, suggesting that compensatory changes in *Nrg4*$^{-/-}$ mice occur with age. Our findings show that NRG4 is a major novel regulator of dendritic arborisation in the developing cerebral cortex and suggest that it exerts its effects by an autocrine/paracrine mechanism.

1. Introduction

The neuregulins are widely expressed pleiotropic growth factors related to epidermal growth factor that signal *via* the ErbB family of receptor tyrosine kinases (Britsch, 2007). Very extensive work on the first neuregulin discovered (NRG1), has revealed that its numerous isoforms play many roles in the development and function of neurons and glia, including regulating the assembly of neural circuitry, myelination, neurotransmission and synaptic plasticity (Mei and Nave, 2014). Likewise, numerous studies on NRG2 and NRG3 have revealed that they participate in synaptogenesis, synaptic function and aspects of neuronal development (Bartolini et al., 2017; Lee et al., 2015; Vullhorst et al., 2015). Importantly, the *Nrg1*, *Nrg2*, *Nrg3*, *ErbB3* and *ErbB4* genes have been identified as susceptibility genes for schizophrenia, depression and bipolar disorder (Mei and Nave, 2014; Rico and Marin, 2011) and numerous genetic and functional studies have directly implicated the *Nrg1*, *Nrg2*, *Nrg3* and *ErbB4* genes in the development of psychotic behaviour (Hayes et al., 2016; O'Tuathaigh et al., 2007, 2010; Shamir et al., 2012; Yan et al., 2017). Although much less work has been done on the latest neuregulins to be identified, NRG5 and NRG6, both are highly expressed in brain (Kanemoto et al., 2001; Kinugasa et al., 2004). NR6 plays a role in radial neuronal migration in the neocortex (Zhang et al., 2013) and is a potential susceptibility gene for schizophrenia (So et al., 2010).

In contrast with other neuregulins, NRG4 is expressed in a limited number of adult tissues, such as brown adipose tissue, and has been reported to have no or negligible expression in adult brain (Harari et al., 1999; Rosell et al., 2014). NRG4 functions as a secreted endocrine factor *in vivo* produced and released by brown adipose tissue. NRG4 decreases hepatic lipogenesis, increases fatty acid β-oxidation and increases energy expenditure (Chen et al., 2017; Wang et al., 2014). While NRG4 has been implicated in the regulation of metabolic homeostasis, it has no known function in the brain. Our analysis of mice in which the *Nrg4* locus has been disrupted reveals a very striking phenotype in neocortical pyramidal neurons both *in vitro* and *in vivo*. As such, we provide the first evidence that NRG4 plays a major role in the brain.

* Corresponding author.
E-mail address: daviesalun@cf.ac.uk (A.M. Davies).

https://doi.org/10.1016/j.expneurol.2018.01.002
Received 29 September 2017; Received in revised form 12 December 2017; Accepted 2 January 2018
Available online 06 January 2018

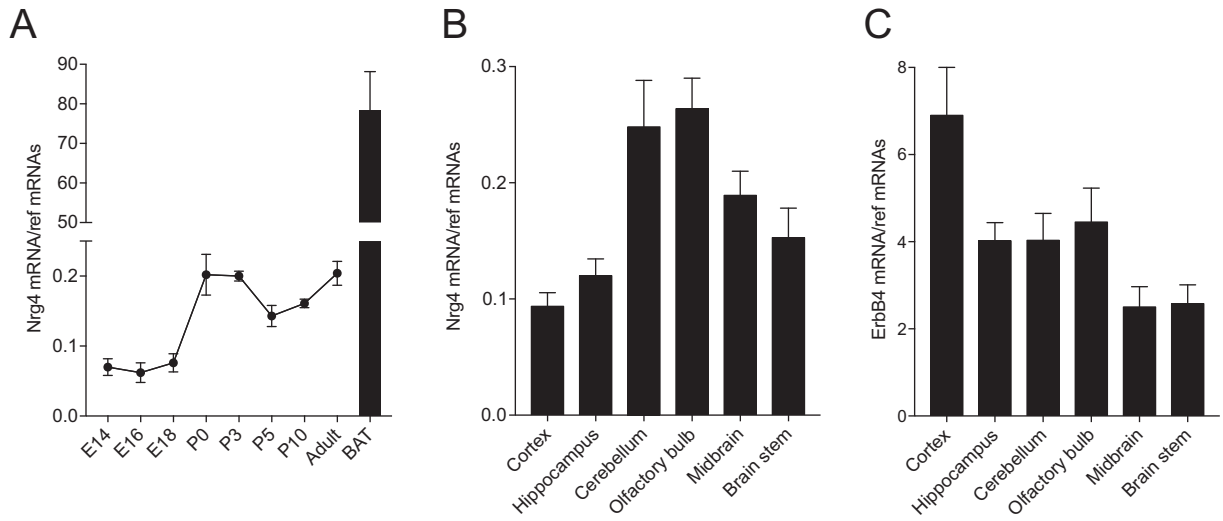

Fig. 1. *Nrg4* mRNA is expressed in the developing brain. Levels of *Nrg4* mRNA in the neocortex at different ages compared with adult brown adipose tissue (BAT) (A) and in different brain regions at P0 (B) and the levels of *ErbB4* mRNA in these regions (C) relative to the geometric mean of reference mRNAs. The mean ± s.e.m. of data from four separate sets of tissues at each age/region are plotted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Animals

Mice were housed in a 12 h light-dark cycle with access to food and water *ad libitum*. Breeding was approved by the Cardiff University Ethical Review Board and was performed within the guidelines of the Home Office Animals (Scientific Procedures) Act, 1986. *Nrg4* null mice in which the *Nrg4* locus was disrupted by retroviral insertion of a gene trap between exons 1 and 2 were purchased from the Mutant Mouse Resource Centre, UC Davis (California, USA). These mice were back-crossed from a C57/BL6 background into a CD1 background. $Nrg4^{+/-}$ mice were crossed to generate $Nrg4^{+/+}$ and $Nrg4^{-/-}$ littermates.

2.2. Neuron culture

Primary cortical neurons were prepared from E16 embryos. The protocol for culturing hippocampal pyramidal neurons was used with modifications (Kaech and Banker, 2006). Briefly, dissected cortices were mechanically triturated in Neurobasal A medium supplemented with 2% B27 (Gibco), 0.5 mM GlutaMAX 1, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). 15,000 cells/cm^2 were plated on poly-L-Lysine (Sigma)-coated 35 mm dishes and incubated at 37 °C in a humidified atmosphere with 5% CO_2. In some cultures, the culture medium was supplemented with 100 ng/ml recombinant NRG4 (Thermo Fisher Scientific) after plating. Neurons were cultured for either 3 or 9 days *in vitro*. Neurons were fluorescently labelled in 3 day cultures by treating the cultures with the fluorescent dye calcein-AM (2 μg/ml, Invitrogen) for 15 min at 37 °C. In 9 days cultures, the neurite arbors of a subset of the neurons were visualized by transfecting the neurons with a GFP expression plasmid using lipofectamine 2000 (Invitrogen, Paisley, UK) after 7 days *in vitro*. Briefly, 1 μg of DNA was mixed with 2 μl of lipofectamine. After 20 min, this mixture in 2 ml of Opti-MEM media (Gibco) was added to the cultures. After 3 h at 37 °C, the cultures were washed with culture medium and incubated for a further 2 days. At the end of the experiment, the neurons were fixed for 30 min with 4% paraformaldehyde. Images of fluorescent-labelled neurons were acquired with an Axiovert 200 Zeiss fluorescent microscope. Neurite length and Sholl analysis were carried out using Fiji (ImageJ) software with the semi-automated plugin Simple Neurite Tracer (Longair et al., 2011).

2.3. Immunohistochemistry

Brains were fixed overnight using 4% paraformaldehyde in 0.12 M phosphate-buffered saline (PBS) at 4 °C, washed in PBS and cryoprotected in 30% sucrose before being frozen in dry ice-cooled isopentane. Serial 30 μm sections were blocked in 1% BSA (Sigma), 0.1% Triton (Sigma) in PBS and then incubated with 1:500 anti-MAP2 (Millipore) and anti-NRG4 (Abcam) antibodies at 4 °C overnight. After washing, the sections were incubated with 1:500 rabbit polyclonal Alexa-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Sections were washed, incubated with DAPI and visualized using a Zeiss LSM710 confocal microscope.

2.4. Immunocytochemistry

Neurons were fixed for 10 mins in 4% paraformaldehyde in 0.12 M phosphate-buffered saline (PBS), washed 3 times in PBS and blocked in 1% BSA (Sigma), 0.1% Triton (Sigma) in PBS for 1 h, then incubated with primary antibodies (1:50) against NRG4 (Santa Cruz), ErbB4 (Abcam), Emx1 (Santa Cruz) overnight at 4 °C. After washing, the neurons were incubated with polyclonal Alexa-conjugated secondary antibodies (Invitrogen) 1:500 for 1 h at room temperature. Cells were then washed, incubated with DAPI (1:8000) and visualized using a Zeiss LSM710 confocal microscope.

2.5. Quantitative PCR

The levels of *Nrg4* mRNA was quantified by RT-qPCR relative to a geometric mean of mRNAs for the house keeping enzymes glyceraldehyde phosphate dehydrogenase (*Gapdh*), succinate dehydrogenase (*Sdha*) and hypoxanthine phosphoribosyltransferase-1 (*Hprt1*). Total RNA was extracted from dissected tissues with the RNeasy Mini Lipid extraction kit (Qiagen, Crawely, UK). 5 μl total RNA was reverse transcribed, for 1 h at 45 °C, using the Affinity Script kit (Agilent, Berkshire, UK) in a 25 μl reaction according to the manufacturer's instructions. 2 μl of cDNA was amplified in a 20 μl reaction volume using Brilliant III ultrafast qPCR master mix reagents (Agilent Technologies). PCR products were detected using dual-labelled (FAM/BHQ1) hybridization probes specific to each of the cDNAs (MWG/Eurofins, Ebersberg, Germany). The PCR primers were: *Nrg4* forward: 5′-GAG ACA AAC AAT ACC AGA AC-3′ and reverse: 5′-GGA CTG CCA TAG AAA TGA-3′; *ErbB4* forward: 5′-GGC AAT ATC TAC ATC ACT G-3′ and reverse: 5′-CCA ACA