Neurobiology of Disease 39 (2010) 292–300

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Satellite glia not DRG neurons constitutively activate EGFR but EGFR inactivation is not correlated with axon regeneration

Zubair Ahmed *, Martin L. Read, Martin Berry, Ann Logan

Molecular Neuroscience Group, Neuropharmacology and Neurobiology Section, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, B15 2TT, UK

ARTICLE INFO

Article history:
Received 10 December 2009
Revised 12 April 2010
Accepted 26 April 2010
Available online 6 May 2010

Keywords:
EGFR
DRGN
Axon/neurite growth
siRNA
DRG satellite glia

ABSTRACT

To test the possibility that phosphorylated epidermal growth factor receptor (pEGFR) mediates axon growth inhibition, we determined if pEGFR levels were raised in dorsal root ganglia (DRG) after non-regenerating dorsal column (DC) lesions and suppressed in regenerating sciatic nerve (SN) and preconditioning (P) SN + DC lesioned DRG. Levels of EGFR mRNA and protein in DRG were unchanged between control and all injury models. Satellite glia and not DRG neurons (DRGN) constitutively contained pEGFR and, only in PSN + DC rats, were levels significantly reduced in these cells. *In vitro*, siRNA mediated knockdown of EGFR (siEGFR) mRNA and protein was associated with suppressed RhoA activation, but fibroblast growth factor-2 (FGF2) was a mandatory requirement for DRGN neuritogenesis after addition of inhibitory concentrations of CNS myelin. Thus, EGFR activation in satellite glia was not consistently correlated with DRGN axogenesis and siEGFR reduction of pEGFR with attenuated Rho-GTP signalling did not promote DRGN disinhibited neurite outgrowth without exogenous FGF2 stimulation. Together, these data argue against a direct intra-axonal involvement of pEGFR in axon regeneration.

Introduction

After dorsal column (DC) lesions, the growth of all centrally projecting dorsal root ganglion neuron (DRGN) axons is arrested at the injury site by both CNS myelin-/scar-derived axon growth inhibitory ligands (e.g. Nogo-A, myelin associated glycoprotein (MAG), chondroitin sulphate proteoglycan (CSPG), ephrins and semaphorins) and a limited availability of neurotrophic factors required to maintain DRGN survival and promote axon regeneration (Hunt et al., 2002; Sandvig et al., 2004; Fournier et al., 2002; McKerracher, 2001; McKerracher and Winton, 2002; Hou et al., 2008; Fabes et al., 2007; Du et al., 2007; Berry et al., 2008). However, a preconditioning (P) lesion to the sciatic nerve (SN), 1–2 w before DC transection, promotes DRGN axonal regeneration (Neumann and Woolf, 1999; Chong et al., 1999). The *in vitro* growth potential of DRGN neurites is also increased after PSN lesions, suggesting that conditioning enhances the intrinsic growth of axotomised DRGN and leads to limited axon regeneration through the non-permissive DC neuropil (Neumann and Woolf, 1999).

The epidermal growth factor receptor (EGFR) has been implicated in mediating disinhibited CNS axon regeneration using the specific reversible and irreversible EGFR kinase antagonists AG1478 and PD168393, respectively (Koprivica et al., 2005; Erschbamer et al., 2007). For example, in the injured optic nerve, PD168393 promotes retinal ganglion cell (RGC) axon regeneration (Koprivica et al., 2005) and rescues RGC from death in a chronic glaucoma model (Liu et al., 2006). In the latter study, EGFR expression was localised to astrocytes, not to RGC or their axons, suggesting that the beneficial effects of EGFR inhibition may be mediated by glia and not neurons. EGFR activation triggers quiescent astrocytes into a reactive phenotype (Liu et al., 2006) that secrete CSPG (Smith and Strunz, 2005) and form a cribriform lattice with their processes that may contribute to the formation of glial scars formed after penetrant injury (Liu and Neufeld, 2004). EGFR is not expressed in mature astrocytes (Gomezpinilla et al., 1988) except after ischemia (Planas et al., 1998; Jin et al., 2002), spinal cord tractotomy (Lisovoski et al., 1997), and in the optic nerve of patients with glaucoma (Liu and Neufeld, 2003; Liu et al., 2006). After spinal cord injury, inhibition of EGFR improves motor and sensory function as well as bladder emptying (Erschbamer et al., 2007). In adult primary DRG and retinal cultures with added CNS myelin extracts (CME), DRGN and RGC neurite outgrowth is induced through the off-target release of neurotrophins from both glia and neurons and raised cAMP levels after AG1478 treatment (Ahmed et al., 2009a; Douglas et al., 2009). Thus, these latter studies assert that EGFR does not directly mediate the inhibition of CNS axon regeneration.

In the present study, we investigated the *in vivo* role of EGFR in adult rat DRGN axon regeneration in a non-regenerating transected DC model, a regenerating SN lesion model, and in a paradigm in which DC axons were stimulated to regenerate after PSN lesions, predicting that neuronal pEGFR levels would be raised in the non-regenerating,

* Corresponding author. Molecular Neuroscience Group, Neuropharmacology and Neurobiology Section, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Room WX2.17, Institute of Biomedical Research (West), Edgbaston, Birmingham B15 2TT, UK. Fax: +44 121 414 8867.
E-mail address: z.ahmed.1@bham.ac.uk (Z. Ahmed).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2010.04.013

but reduced in the regenerating models if an intra-axonal role is valid. In primary DRG cultures, we used an siRNA to EGFR (siEGFR) to reduce EGFR titres, predicting that siEGFR knockdown would promote disinhibited DRGN neurite outgrowth in the presence of CME, similar to that induced by AG1478 and PD168393 (Koprivica et al., 2005). In agreement with our previous findings (Ahmed et al., 2009a; Douglas et al., 2009), the results of the current experiments provide no support for direct intra-axonal negative regulation of neurite/axon growth through the EGFR signalling axis.

Materials and methods

In vivo experimental design

The experiments comprised 4 groups of animals each containing 10 adult male Sprague–Dawley rats (150–200 g) (Charles River, Margate, UK) for each analytical end-point ($n = 10$ DRG), designated as: (1), uninjured controls; (2) DC crush (non-regenerating DC lesions); (3), SN crush (regenerating SN lesions); and (4), PSN lesion 1 w before a DC crush (regenerating PSN + DC lesions).

Surgical procedures

All surgical procedures were licensed by the UK Home Office, approved by the University of Birmingham Ethical Committee and conducted under inhalation anaesthesia induced with 5% isofluorane with 1.5 L/min O_2 and maintained with reducing levels of isofluorane. The DC was crushed bilaterally at the level of T8 using calibrated watchmaker's forceps inserted through the dorsal cord meninges to a depth of 1.5 mm (Lagord et al., 2002). The left SN was exposed at a mid-thigh level and crushed using suture forceps at the level of the sacrotuberous ligament. A preconditioning lesion of the SN was performed 1 w before DC crush, as described above. Animals were killed by CO_2 exposure 10 d after surgery and the L4/L5 DRG was harvested for RNA and protein analysis by snap freezing in liquid nitrogen. The contralateral L4/L5 DRG served as uninjured controls. For immunohistochemistry, animals were intracardially perfused with 4% formaldehyde and processed as described later.

Microarray analysis

The rat genome AROS™ V3.0 set (Operon Biotechnologies GmbH, Cologne, Germany) contained 26,962 long mer probes representing 22,012 genes and 27,044 gene transcripts. Slide preparation was performed by the Functional Genomics Laboratory (University Of Birmingham, UK). Oligonucleotides were resuspended in Pronto Universal Slide Spotting Solution (Fisher Scientific, Loughborough, UK) and subsequently spotted onto UltraGAPS Coated Slides with Bar Code (Fisher Scientific) using a BioRobotics Microgrid II spotter (Genomic Solutions Ltd, Huntingdon, UK). Extracted total RNA (0.5–1 μg) was amplified using the Amino Allyl Message Amp II aRNA kit (Ambion, Austin, TX, USA) and then labelled with either Cy3, or Cy5 dyes (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. The frequency of label incorporation was calculated using the dye incorporation calculator (www.ambion.com/tools/dye), and frequencies of 30–60 dye molecules/1000 nucleotides were considered a successful coupling reaction. Probes were resuspended in long oligo Pronto hybridisation buffer (Corning, Lowell, MA, USA) with mouse Hybloc solution (Insight Biotechnology, London, UK) before hybridization. Microarray hybridisation used a Pronto hybridisation kit (Corning) according to the manufacturer's protocol. The slides were scanned with an Axon GenePix® 4000B scanner (Molecular Devices Ltd, Berkshire, UK) set at 600 V and images were analysed using GenePix® V5.0 software (Molecular Devices). Each experiment was replicated at least ×4. Normalisation of the microarray data and subsequent analyses were performed using GeneSpring GX7 software (Agilent Technologies, Cheshire, UK). In this study, we selected neuropeptide Y (*npy*), galanin (*gal*), activating transcription factor 3 (*atf3*) and growth associated protein 43 (*gap43*) as our markers for a regenerative phenotype in DRGN (Costigan et al., 2002) and *egfr* mRNA for further analysis.

Real-time RT-PCR

Ipsilateral and contralateral to the crushed SN, L4/L5 DRG were dissected and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Purified RNA was reverse-transcribed into cDNA using the Reverse Transcription System (Promega, Southampton, UK) according to the manufacturer's protocol. PCR reactions were set up in ABI PRIZM™ 96 well optical reaction plates using 40 ng of the cDNA template, 2× Universal PCR Mastermix and either 18S endogenous control probe, or the relevant Taqman Gene Expression Assay (all from Applied Biosystems, Foster City, CA, USA), and amplified on an ABI PRIZM® 7700 (Applied Biosystems) set at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and then at 60 °C for 1 min. PCR data were collected and the relative expression of target genes was calculated using the $2^{\Delta\Delta Ct}$ method (DataAssist™ Software, Applied Biosystems). Taqman Gene Expression Assays (Applied Biosystems) were used to measure relative mRNA levels of the axon regeneration related genes *npy*, *gal*, *atf3*, *gap43* (Costigan et al., 2002) and compared with *egfr* expression.

Antibodies

Polyclonal rabbit anti-human EGFR and polyclonal goat anti-human pEGFR (Santa Cruz Biotechnology, CA, USA) were used to localise total EGFR and phosphorylated EGFR in immunohistochemistry/immunocytochemistry both at 1:200 dilution. Blocking peptides for EGFR and pEGFR (Santa Cruz Biotechnology, CA, USA) were used at 1:20 concentration to confirm antibody specificities. Monoclonal β-III tubulin antibody (Sigma, Poole, UK) labelled DRGN neurites at 1:200 by immunocytochemistry. For western blotting both EGFR and pEGFR were used at 1:500 dilution, while total Rho was detected using a monoclonal anti-Rho antibody diluted at 1:200 (Upstate Biotechnology, Milton Keynes, UK) and β-actin (Sigma) at 1:10,000 dilution as a loading control for western blots.

Adult DRGN cultures

L4-L7 DRG pairs were dissected from 6 to 8 w-old Sprague–Dawley rats and dissociated into single cells using a solution of Neurobasal-A (Invitrogen, Paisley, UK) containing 0.1% collagenase (Sigma) and 200 U/ml DNaseI (Worthington Biochem, New Jersey, USA) as previously described by us (Ahmed et al., 2009a). DRGN were cultured at 500 cells/chamber on sterile glass chamber slides (BD Biosciences, Oxford, UK) pre-coated with 100 μg/ml poly-D-lysine followed by 20 μg/ml Laminin-I (Sigma), in supplemented Neurobasal-A medium, in both the presence and absence of the same batch of rat CNS myelin known to contain Nogo-A, OMgp, MAG and CSPG (Ahmed et al., 2005), for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were also treated with 30 μM forskolin (Sigma) to raise cAMP levels and fibroblast growth factor-2 (FGF2) (Peprotech, London, UK) to promote DRGN neurite outgrowth (Ahmed et al., 2005) where appropriate.

Immunohistochemistry

Animals were killed 10 d post injury (dpi) and intracardially perfused with 4% formaldehyde (TAAB Laboratories, Berkshire, UK). L4/L5 DRG were then post-fixed in 4% formaldehyde, cryoprotected through a graded series of sucrose solutions and blocked up in OCT mounting compound (TAAB Laboratories). Sections of DRG were cut