Available online at www.sciencedirect.com
ScienceDirect
Neurochemistry International 50 (2007) 491–498
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

BDNF production by olfactory ensheathing cells contributes to axonal regeneration of cultured adult CNS neurons

Erika Pastrana [a], Maria Teresa Moreno-Flores [a], Jesus Avila [a], Francisco Wandosell [a], Liliana Minichiello [b], Javier Diaz-Nido [a,*]

[a] *Centro de Biologia Molecular "Severo Ochoa", Universidad Autonoma de Madrid, Madrid, Spain*
[b] *European Molecular Biology Laboratory (EMBL), Monterontondo, Italy*

Received 5 May 2006; received in revised form 5 October 2006; accepted 10 October 2006
Available online 8 December 2006

Abstract

Olfactory ensheathing cells (OECs) are the main glial cell type that populates mammalian olfactory nerves. These cells have a great capacity to promote the regeneration of axons when transplanted into the injured adult mammalian CNS. However, little is still known about the molecular mechanisms they employ in mediating such a task. Brain-derived neurotrophic factor (BDNF) was identified as a candidate molecule in a genomic study that compared three functionally different OEC populations: Early passage OECs (OEC Ep), Late passage OECs (OEC Lp) and the OEC cell line TEG3 [Pastrana, E., Moreno-Flores, M.T., Gurzov, E.N., Avila, J., Wandosell, F., Diaz-Nido, J., 2006. Genes associated with adult axon regeneration promoted by olfactory ensheathing cells: a new role for matrix metalloproteinase 2. J. Neurosci. 26, 5347–5359]. We have here set out to determine the role played by BDNF in the stimulation of axon outgrowth by OECs. We compared the extracellular BDNF levels in the three OEC populations and show that it is produced in significant amounts by the OECs that can stimulate axon regeneration in adult retinal neurons (OEC Ep and TEG3) but it is absent from the extracellular medium of OEC Lp cells which lack this capacity. Blocking BDNF signalling impaired axonal regeneration of adult retinal neurons co-cultured with TEG3 cells and adding BDNF increased the proportion of adult neurons that regenerate their axons on OEC Lp monolayers. Combining BDNF with other extracellular proteins such as Matrix Metalloproteinase 2 (MMP2) further augmented this effect. This study shows that BDNF production by OECs plays a direct role in the promotion of axon regeneration of adult CNS neurons.

Keywords: Olfactory ensheathing cells; Axonal regeneration; Brain-derived neurotrophic factor; Neurotrophin; Retinal ganglion cell; Genomic analysis

1. Introduction

Olfactory ensheathing cells (OECs) are the main glial cell type associated with the mammalian olfactory nerve. In vivo, they accompany and surround the axons of olfactory receptor neurons from the point at which they emerge in the olfactory epithelium to the olfactory bulb (Boyd et al., 2003). Indeed, OECs are thought to be responsible for the continuous growth of new axons into the CNS tissue that takes place in the adult mammalian olfactory systems (Doucette, 1990). For this reason, many attempts to repair damage in the injured CNS have relied on the use of OEC grafts (Moreno-Flores et al., 2002; Barnett and Chang, 2004).

It has been demonstrated that OECs have the capacity to promote the regeneration of CNS axons both in vivo and in vitro (Chuah and West, 2002; Moreno-Flores et al., 2002; Wewetzer et al., 2002; Boyd et al., 2003; Barnett and Chang, 2004). In vivo, olfactory ensheathing cell grafts have been shown to promote axon regeneration and neurite sprouting in injured dorsal root ganglion neurons, cortico-spinal neurons and optic nerve axons (Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 1997, 2003a,b; Raisman, 2000; Ramon-Cueto et al., 2000; Bunge, 2002; Chuah et al., 2004; Moreno-Flores et al., 2006). Furthermore, olfactory receptor neurons, cortical neurons, hippocampal neurons, retinal ganglion cells (RGCs), granule cerebellar neurons and sympathetic and dorsal root ganglion neurons are all capable of regenerating their axons in vitro when cultured on OEC monolayers (Goodman et al., 1993; Chuah and Au, 1994; Kafitz and Greer, 1999; Sonigra et al., 1999; Lipson et al., 2003; Moreno-Flores et al., 2003a;

* Corresponding author. Tel.: +34 91 497 8475; fax: +34 91 497 4799.
E-mail address: javier.diaznido@uam.es (J. Diaz-Nido).

0197-0186/$ – see front matter
doi:10.1016/j.neuint.2006.10.004

van den Pol and Santarelli, 2003; Chung et al., 2004; Deumens et al., 2004; Pastrana et al., 2006).

Despite the advances in our understanding of the functional properties of OECs, there is still little known about the molecular mechanisms through which OECs mediate axonal repair (Chuah and West, 2002; Keyvan-Fouladi et al., 2002). In this line, a recent study has sought to compare the genetic expression profiles of three populations of olfactory ensheathing cells that differ in their capacity to stimulate the growth of adult CNS axons in vitro (Pastrana et al., 2006). This analysis led to the detection of a number of candidate molecules whose expression correlates with the functional capacity of OECs to stimulate axon outgrowth. One of the molecules detected in the genomic study was brain-derived neurotrophic factor (BDNF). BDNF is part of the neurotrophin family of secreted proteins, which are essential for the development and functioning of the vertebrate nervous system. BDNF can signal through two types of cell-surface receptors: the tyrosine kinase receptor TrkB and the p75 pan-neurotrophin receptor. Several studies have previously shown that olfactory ensheathing cells produce BDNF and other neurotrophic factors in vitro (Woodhall et al., 2001; Lipson et al., 2003). Furthermore, BDNF has been found in vivo in association with olfactory ensheathing cell grafts in the injured rat spinal cord (Sasaki et al., 2006). However, direct evidence demonstrating the implication of BDNF in the stimulation of axon outgrowth by OECs is still lacking.

In this study, we set out to determine the implication of BDNF signalling in the promotion of axon regeneration elicited by olfactory ensheathing cells. Employing an in vitro OEC functional assay, we have determined the role played by BDNF in mediating axon regeneration in adult retinal ganglion neurons. This neuronal population has been broadly used in studies regarding the implication of BDNF in axon outgrowth since it is well characterized that these cells respond to BDNF and express the high-affinity receptor TrkB both under normal conditions and after injury (Cui et al., 2002; Goldberg et al., 2002). Altogether, we show here that BDNF production by OECs is implicated in stimulating the regeneration of adult CNS axons.

2. Experimental procedures

2.1. Experimental animals

All animals were handled according to European Union (86/609/EEC) guidelines and special care was taken to minimise animal suffering and the number of animals used. Postnatal day (p) 21 and 2-month-old rats and mice were deeply anesthetized with CO_2 and sacrificed to obtain tissue for the cultures.

2.2. Olfactory ensheathing cell cultures

Olfactory ensheathing cell cultures were performed and maintained as described previously (Pastrana et al., 2006). Primary olfactory ensheathing cells (OEC Ep) were prepared from p21 rat olfactory bulbs following previously described procedures (Moreno-Flores et al., 2003b). Dissociated cells were plated onto 10 μg/ml poly-L-lysine (Sigma, St. Louis, MO) coated dishes and maintained in M3 culture medium. M3 contains Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 3% fetal calf serum (FCS), 20 μg/ml bovine pituitary extract (Sigma) and 1 μM forskolin (Sigma). The cells were maintained at 37 °C with 5% CO_2 for 1 week in culture and are named Early passage OECs or OEC Ep cells. Olfactory ensheathing cell purity in these cultures was determined by performing immunocytochemical studies with various well-known OEC protein markers (see Fig. 1 and Pastrana et al., 2006). As is shown in Fig. 1, between 85 and 90% of the cells in our primary cultures were OECs, as defined by the expression of the OEC markers 3-phosphoglycerate dehydrogenase (3PGDH), s100β, nestin and the neurotrophin receptor p75 (p75NTR).

Primary OEC Ep cultures were maintained for 1 week in culture until they reached confluence, at that time, neurons were plated onto glial monolayers to perform functional studies (Pastrana et al., 2006).

The population of OECs adapted to long-term culture (OEC Lp) was obtained by serial passage of a primary culture of olfactory ensheathing cells in M3 medium (Pastrana et al., 2006). Cells were maintained for over 1 year in vitro (surpassing 50 population doublings). Functional studies of this population were performed as described above for primary OEC cultures.

The clonal cell line TEG3, obtained by immortalization using the SV40 large T antigen (Moreno-Flores et al., 2003a,b), was cultured in M3 medium as described above for the other OEC populations.

2.3. In vitro assay of axonal regeneration

Axonal regeneration assays were performed and analysed as previously described (Pastrana et al., 2006). Retinal tissue was extracted from 2-month old rats and 2-month old wild-type and transgenic TrkB$^{shc/shc}$ mice (Minichiello et al., 1998, 2002). Retinal tissue was then digested with papain using Worthington's Papain System (20 units/ml; Worthington-Biochemical Corporation, Lakewood, NJ). Axotomized adult 2-month-old retinal neurons were plated on OEC monolayers and the cultures were maintained in serum-free Neurobasal medium (Gibco-BRL), supplemented with B-27 (Gibco-BRL) and 12.5 mM KCl for 50 h at 37 °C with 5% CO_2 before fixing with 4% paraformaldehyde (PFA).

Axonal regeneration events were thoroughly characterized and quantified as described previously (Pastrana et al., 2006). Co-cutures were fixed after 50 h in vitro and analysed by immunocytochemistry employing an antibody against the phosphorylated forms of the microtubule-associated protein 1B (MAP1B) and Neurofilament-H (NF-H) protein (SMI31; Sternberger Monoclonal Inc., Baltimore, MD; 1:500) and an antibody which recognizes high molecular weight microtubule-associated protein 2 (MAP2; 514; Sanchez Martin et al., 1998; 1:400). The percentage of retinal neurons with an axon was determined by counting the number of MAP2 positive neurons that bear a polarised neurite that can be labelled with antibodies against the phosphorylated forms of MAP1B and NF-H proteins. Blind quantifications were performed under the 40× objective of an inverted Axiovert200 microscope (Zeiss, Oberkochen, Germany).

2.3.1. Treatments

The following reagents were administered to the co-cultures immediately after plating the neurons: BDNF neutralizing antibody (IgY isotype), control IgY antibody (both used at a concentration of 20 μg/ml and obtained from Promega, Madison, USA), anti-NGF neutralizing antibody (Sigma; 20 μg/ml), TrkB tyrosine kinase inhibitor k-252a (10 nM; Calbiochem, Germany), recombinant BDNF (50 ng/ml, Alomone labs, Jerusalem, Israel) and recombinant MMP2 protein (0.2 μg/ml; Biomol, Plymouth Meeting, PA).

2.4. Immunocytochemistry

Immunostainings were performed as follows. Cells cultured on cover-slips were fixed with 4% PFA, washed several times in PBS and blocked with PBS containing 0.1%Triton X-100 and 1% serum (PBS-TS). Cover-slips were then incubated at room temperature for 2–3 h in PBS-TS containing the diluted primary antibodies. After washing, the cells were incubated for another hour in a solution of PBS-TS containing the secondary antibodies conjugated to fluorescein isothiocyanate (FITC; Jackson Labs., West Groove, PA), 488 or Alexa (Molecular Probes, Eugene, OR, USA). Nuclear staining was performed by incubating the preparations for 10 min in a solution of PBS with DAPI (Calbiochem, Germany; 1:10,000). Finally, the cover-slips were washed and mounted with fluoromount (Southern Biotech., Birmingham, AL). The labelled preparations were visualised using an inverted Axiovert200 (Zeiss) microscope coupled to a CCD camera (Spot ST, Slider).