ELSEVIER

Molecular Brain Research 88 (2001) 203–213

BRAIN RESEARCH INTERACTIVE

www.elsevier.com/locate/bres

Interactive report

Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors[1]

Emma Woodhall[a], Adrian K. West[b], Meng Inn Chuah[a,*]

[a]*Department of Anatomy and Physiology, University of Tasmania, P.O. Box 252-24, Hobart, Tasmania 7001, Australia*
[b]*Department of Biochemistry, University of Tasmania, Hobart, Tasmania 7001, Australia*

Accepted 5 February 2001

Abstract

In the primary olfactory pathway axons of olfactory neurons (ONs) are accompanied by ensheathing cells (ECs) as the fibres course towards the olfactory bulb. Ensheathing cells are thought to play an important role in promoting and guiding olfactory axons to their appropriate target. In recent years, studies have shown that transplants of ECs into lesions in the central nervous system (CNS) are able to stimulate the growth of axons and in some cases restore functional connections. In an attempt to identify a possible mechanism underlying EC support for olfactory nerve growth and CNS axonal regeneration, this study investigated the production of growth factors and expression of corresponding receptors by these cells. Three techniques immunohistochemistry, enzyme linked immunosorbent assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to assess growth factor expression in cultured ECs. Immunohistochemistry showed that ECs expressed nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF). ELISA confirmed the intracellular presence of NGF and BDNF and showed that, compared to BDNF, about seven times as much NGF was secreted by ECs. RT-PCR analysis demonstrated expression of mRNA for NGF, BDNF, GDNF and neurturin (NTN). In addition, ECs also expressed the receptors trkB, GFRα-1 and GFRα-2. The results of the experiments show that ECs express a number of growth factors and that BDNF in particular could act both in a paracrine and autocrine manner.

Theme: Development and regeneration

Topic: Glia and other non-neuronal cells

Keywords: Olfactory ensheathing cells; Growth factors; Nerve growth factor; Brain derived neurotrophic factor; Glia cell line-derived neurotrophic factor; Cell culture

1. Introduction

The olfactory neuroepithelium is one of the few sites in the mammalian nervous system that is capable of neurogenesis throughout life [25,53]. When olfactory neurons (ONs) die, new neurons are produced by division and differentiation of basal cells that reside in the olfactory epithelium [19,25,34]. These newly formed neurons grow axons and are able to make the right synaptic connection with their target neurons in the olfactory bulb (OB) which is part of the central nervous system (CNS). The permissivity of the adult OB to the growth of axons persists after transection of olfactory nerves [16,24] or lesioning of the olfactory nerve layer of the bulb [16]. This capacity is thought to be due in part to the unique glial cells of the olfactory nerve, known as olfactory ensheathing cells (ECs). These cells share phenotypic characteristics in common with Schwann cells and astrocytes [5,15,41,47] but are unique in that they can cross the transitional zone between the peripheral nervous system and CNS [17,45]. Although it is well documented that ECs express extracellular matrix molecules such as N-CAM and laminin [22,33] that could contribute to axonal growth, few studies

[1]Published on the World Wide Web on 6 March 2001.

*Corresponding author. Tel.: +61-3-6226-2664; fax: 61-3-6226-2679.
E-mail address: inn.chuah@utas.edu.au (M.I. Chuah).

0169-328X/01/$ – see front matter
PII: S0169-328X(01)00044-4

have shown unequivocal direct evidence that ECs synthesize and secrete growth factors [13].

Several studies have shown that transplants of ECs in nerve lesion sites stimulate the outgrowth of neurites and in some cases restore functional connections [31,39,46, 48,56]. The underlying assumption is that the ECs are probably facilitating repair by secreting growth-promoting molecules such as extracellular matrix molecules or soluble growth factors. In an attempt to identify a possible mechanism underlying EC support for neurite outgrowth this study investigated the production of specific growth factors and expression of the corresponding receptors by cultured ECs.

The present study investigates the expression of three neurotrophins in ECs, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). The receptors for the neurotrophins include the high affinity tyrosine kinases, trkA, trkB and trkC [4] and the low affinity neurotrophin receptor $p75^{NTR}$ [37]. In addition we investigated the expression of the neurotrophin-related factors, glial cell-line derived neurotrophic factor (GDNF) and neurturin (NTN) in ECs. These factors interact with the GDNF family receptor alpha-1 (GFRα-1), GFRα-2 (which determine ligand specificity) and the signal transducing receptor RET [2].

The current studies used immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR) to demonstrate that ECs produce NGF, BDNF, GDNF and NTN. Ensheathing cells were also shown to express receptors for these growth factors suggesting that they may be acting in an autocrine manner in addition to a paracrine action in promoting olfactory neuron differentiation.

2. Materials and methods

2.1. Olfactory ensheathing cell culture

Highly enriched cultures of ECs were prepared from the OBs of 2-day-old hooded Wistar rats according to the method described by Chuah and Teague [12]. Animals were anesthetised by intraperitoneal injection of 0.1 ml of pentobarbitone sodium (60 mg/kg) (Boehringer Ingelheim, Artarmon, NSW, Australia) diluted 1:20 in normal saline prior to decapitation. In summary, the olfactory nerve layer was peeled away from the rest of the bulb and digested with 0.25% trypsin and 0.03% collagenase to dissociate the cells. Contaminating cells such as fibroblasts were removed by treatment with cytosine arabinoside (2.5×10^{-7} M). Ensheathing cells were then enriched by addition of bovine pituitary extract (BPE) (12 μg/ml) (Sigma, St. Louis, MO) to the culture medium. After 2–3 days the cells had reached confluency and were passaged to a new flask to eliminate any remaining fibroblasts. Once the cells had again reached confluency they were trypsinized and used for ELISA, RT-PCR, or immunohistochemical staining.

Ensheathing cells are known to express several antigenic molecules including $p75^{NTR}$ [20,23,61] and S-100 protein [3,44]. In this study double staining with anti-S-100 and Nuclear Yellow (Sigma, St. Louis, MO) was randomly performed in different batches to ascertain the high purity of EC cultures which has been consistently obtained in earlier studies (Fig. 1) [10,12]. Random areas of cells plated on coverslips were photographed with the Olympus PM30 photomicrographic-system and the number of ECs, as indicated by double labeled cells, was counted. This was then expressed as a percentage of the total number of cells. Out of the 207 cells counted in five frames (from three coverslips of different batches of culture), all of them were S-100 positive.

For immunohistochemistry the cells were replated onto acid-cleaned coverslips (1×10^4 cells/13-mm coverslip) and cultured in serum-containing medium (Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 1% minimum essential medium–vitamin solution and 1% penicillin/streptomycin) (Life Technologies, Melbourne, VIC, Australia) for a further 1–2 days.

2.2. Immunohistochemical staining of olfactory ensheathing cells

Immunohistochemistry was used to demonstrate the presence of growth factor proteins and their receptors in ECs. Olfactory EC-covered coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min. After the cells were rinsed in modified Eagle's medium plus HEPES (MEM-H) (Sigma, St. Louis, MO), acid alcohol (5% acetic acid; 95% absolute ethanol) was applied and the cells were incubated at −4°C for 15 min to precipitate the protein. The cells were then incubated with polyclonal antibodies against nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and glial cell-line derived neurotrophic factor (GDNF) (1:100, Promega, Madison, WI), followed by the appropriate biotinylated secondary antibody (DAKO, Carpinteria, CA), each for 30 min at room temperature. After washing in MEM-H the cells were incubated for 15 min with the streptavidin–horseradish peroxidase complex (DAKO LSAB+ Kit; DAKO Carpinteria, CA). Finally the cells were washed and reacted for 5 min with 3′,3-diaminobenzidine (DAB) in the presence of hydrogen peroxide. No non-specific staining of ECs was observed in control incubations in which the primary antibody was omitted. Following staining the cells on the coverslips were dehydrated in an ascending series of alcohol, mounted and photographed either with the Olympus PM-30 automatic photomicrographic system or the Olympus DP10 digital camera system. Digital images of the cells were enhanced for contrast and brightness using Adobe Photoshop 5.5.

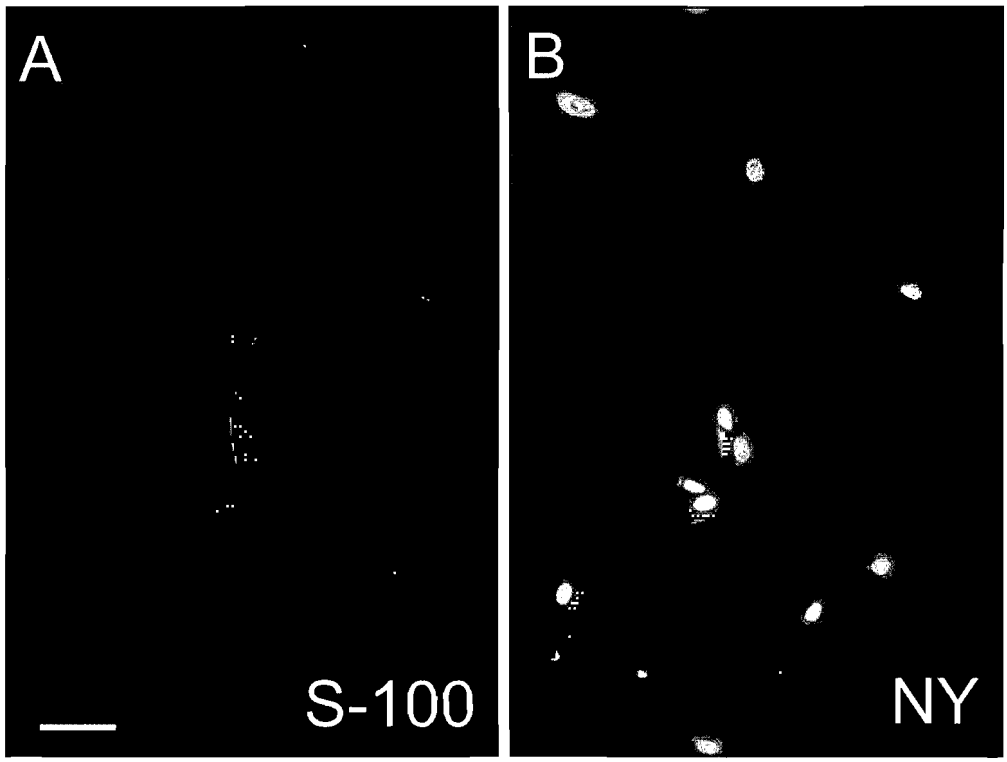

Fig. 1. Double labeling of cells with anti-S-100 protein (A) and Nuclear Yellow (B). All the cells labeled with Nuclear Yellow show immunoreaction for S-100 indicating that they are ensheathing cells. Scale bar: 25 μm.

2.3. Preparation of ensheathing cell conditioned medium

Ensheathing cell conditioned medium was collected and used for analysis of neurotrophic factor secretion by ELISA. Conditioned medium was prepared from confluent EC monolayers prepared as described above. Ensheathing cells were rinsed by three 15-min incubations with Hank's balanced salt solution (Sigma, St. Louis, MO) and incubated in 3 ml serum-free medium developed by Bottenstein and Sato [7] and modified by Chuah and Teague [12]. The serum-free medium contained 561 mg glucose, 0.5 mg bovine insulin, 10 mg transferrin, 6.2×10^{-3} mg progesterone, 1.61 mg putrescine dichloride, 1.587×10^{-2} mg sodium selenite, 0.04 mg thyroxine, 163.9 mg potassium chloride and 1% penicillin/streptomycin per 100 ml of Dulbecco's modified Eagle medium). After 2–3 days the supernatant was collected and concentrated 20 times using a Millipore Ultrafree-15 centrifugal filter (Millipore, Bedford, MA) with a nominal molecular weight limit of 5 kDa, and stored at −20°C for later use.

2.4. Enzyme linked immunosorbent assay (ELISA)

The Emax™ immunoassay system (Promega Madison, WI) was performed on ECs and conditioned medium to determine whether these cells express neurotrophic factor proteins and if they secrete these proteins into the surrounding culture medium.

Prior to each immunoassay, cultured ECs and neonatal rat brain homogenates were acid-treated. This procedure increased the detectable amount of free protein in solution by dissociating the proteins from their proforms or receptors [43]. The acid-treatment procedure was carried out in accordance with the manufacturer's guidelines (Promega, Madison, WI). This process involved diluting the cell suspension 1:5 in lysis buffer (described below) and lowering the pH of the suspension to ~3 with 1 M HCl. After 20 min on ice at 4°C the suspension was neutralised with the appropriate volume of 1 M NaOH and stored at −20°C prior to use.

The procedure for the assay was carried out according to the supplier's instructions. This involved coating a 96-well plate with a polyclonal antibody. Prior to addition of the standard and test samples, the plate was incubated with a blocking solution to minimize any non-specific binding. Standard curves were generated using known amounts of the growth factors and these were used to determine the concentration of respective growth factors in the test samples. Negative controls included lysis buffer (137 mM NaCl, 20 mM Tris pH 8, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 10 μg/ml apoprotinin, 1 μg/ml leupeptin and 0.5 mM sodium vanadate) and serum-free medium, which were used to prepare the EC lysate, brain homogenate and conditioned medium, respectively. Neonatal rat brain homogenate was included as a positive control. The detection system included species-specific monoclonal

antibodies, recognised by polyclonal antibodies and detected by a species-specific anti-IgY antibody conjugated to horseradish peroxidase. Between each incubation the plate was washed with buffer containing 20 mM Tris–HCl, 150 mM NaCl and 0.05% Tween®-20. The chromogenic substrate solution tri-methylbenzoate was then added to the plate followed by 1 M phosphoric acid to stop the reaction. The colour change generated by the oxidation–reduction reaction was measured using a plate reader (Titertek Plus MS212) set at a wavelength of 450 nm. The standard curve for each factor diluted in assay diluent (block and sample buffer) provided a linear curve on a plot of absorbance versus concentration. The minimum detection limit for each assay was 15.6 pg/ml NGF, 15.6 pg/ml BDNF, 10 pg/ml NT-3 and 30 pg/ml GDNF.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

To determine if ECs produced mRNA for NGF, BDNF and NT-3, RT-PCR was performed. This analysis was also used to identify members of the trk receptor family including trkA, trkB and trkC the high affinity receptors for NGF, BDNF and NT-3. The neurotrophin related factors GDNF and NTN were also analysed by RT-PCR along with their receptors, GDNF family receptor alpha-1 (GFRα-1), GFRα-2 and the RET tyrosine kinase receptor. RNA was extracted by the guanidium isothiocyanate and phenol–chloroform method described previously [9]. Samples included ECs, fibroblasts and snap frozen neonatal OBs which had intact olfactory nerve layer. Fibroblasts were isolated from neonatal rat skin according to a method that we have used in an earlier study [13].

RNA was reverse transcribed at 37°C for 1 h using oligo$(dt)_{15}$ primers and murine leukaemia virus (MLV) reverse transcriptase (Promega, Madison, WI). Amplification was performed with 2 μl cDNA, 0.2 μM primers, 10 μM of each dNTP, 1× PCR buffer, 1× Q-solution, 2.5 mM $MgCl_2$ and 2.5 Units of Taq polymerase (Quiagen). Preliminary reactions were carried out at 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, for 37 cycles with a final extension step at 72°C for 5 min using Taq DNA polymerase (Promega, Madison, WI).

We utilised primer sets that have been shown previously to identify alternatively spliced mRNA isoforms for each neurotrophin gene (Table 1). The BDNF primer set was designed to amplify four separate transcripts containing exons 1, 2, 3 or 4 joined to the common exon 5 of the BDNF gene. Unlike the BDNF primer set, which included four separate forward primers and a single reverse primer, the primers for NGF included a single forward and reverse sequence. These primers were specific for sequences in exon 3B and exon 4, which are common to each of the known NGF mRNA isoforms. The primers for NT-3 were aimed at the alternatively spliced exon 1 (which gives rise to isoforms A and B of NT-3) and the common exon 2. These primers produced fragments corresponding to NT-3 1A and 1B.

The trk receptor family frequently express additional polypeptides or truncations in their cytoplasmic domains [27]. The primer sets used to detect the trk receptors in this study were specific for all transcripts of trk, except the truncated forms [40].

GDNF and NTN are grouped together as members of a new TGF-β subfamily called the GDNF family and their proteins demonstrate roughly 42% homology [30]. The primers for GDNF, NTN, GFRα-1, -2 and RET were designed to amplify single fragments. However the GDNF primers have been shown to produce two products perhaps due to alternative splicing of the gene [58].

Primer-template pairs that proved difficult to amplify (i.e. BDNF, GDNF NTN and trk receptors) were repeated using gradient RT-PCR in which eight reactions were prepared for each combination. These reactions were

Table 1
PCR primers and sequences used in the analysis of NGF, BDNF, NT-3, GDNF and their receptors

Target	Forward	Reverse	PCR product (bp)	Reference
NGF	TGG ACC CAA GCT CAC CTC A	GTG GAT GAG CGC GCT TGC TCC T	516	[40]
BDNF Exon 1	ACT CAA AGG GAA ACG TGT CTC T	TCG ATC ACG TGC TCA AAA GTG T	520	[40]
BDNF Exon 2	CGG TGT AGG CTG GAA TAG ACT		400	[40]
BDNF Exon 3	CTC CGC CAT GCA ATT TCC ACT		451	[40]
BDNF Exon 4	GTG ACA ACA ATG TGA CTC CAC T		286	[40]
NT-3 Exon 1A	CAA ACC TCC AAA GTG CTG TGT	GGG GTG AAT TGT AGC GTC TCT	624	[40]
NT-3 Exon 1B	CCC TGG AAA TAG TCA TAC GGA T		371	[40]
GDNF	ATG AAG TTA TGG GAT GTC CTG GCT G	ACC GTT TAG CGG AAT GCT TTC TTA G	660 and 590	[59]
NTN	AGC TCC CTG TAT CTG TCT GGA TGT G	AGG AAG GAC ACC TCG TCC CAT AGG	482	[32]
TrkA	TGG CTG CCT TCG CCT CAA CCA G	ATG GTG GAC ACA GGT ATC ACT G	483	[40]
TrkB	AAT GAC CCA GAG AAC ATC AC	CAG GAA ATG GTC ACA GAC TT	466	[40]
TrkC	CCC TAC ACC TCC TAT CAC TG	CTG GAA ATC CTT CCT GGC AG	516	[40]
GFRα-1	GAA TTC GCA AGT TGG GTC GGA ACT AAC	GTC CAG GCA GTT GTT CCC TTT G	717	[32]
GFRα-2	AAT GAG CTG TGT GCG GCT GAA TC	GCA TGC GGT AGG TAT ACT CGC TGG	528	[32]
RET	CTT GCT CAC CGT CTT CCT CCA G	AGT GCC CTC CTT CCG CTT AAA CTC	525	[32]

cycled at different annealing temperatures in a gradient thermal cycler (PC960G, Corbett Research, Australia) and separated on an agarose gel in order of decreasing annealing temperature.

3. Results

3.1. Ensheathing cells express neurotrophin and GDNF proteins

The primary antibodies used to analyse expression of BDNF, NGF, NT-3 and GDNF were polyclonal antibodies shown to have less than 3% cross-reactivity with structurally related growth factor proteins (Promega technical bulletin). The immunohistochemical analysis demonstrated that ECs were immunoreactive for BDNF, NGF and GDNF. Generally the cells displayed more intense staining for NGF, BDNF and GDNF in the cell bodies and reduced staining in the processes (Fig. 2). Stronger immunoreactivity for BDNF, NGF and GDNF was observed in presumably undifferentiated spherical cells that lacked processes. No evidence for NT-3 immunoreactivity could be found. The negative control, in which the primary antibody was omitted, contained no detectable immunoreactivity.

3.2. Ensheathing cells secrete neurotrophin proteins

The immunoassay system was used to determine whether cultured ECs produced neurotrophic factor proteins and to test if these factors were primarily present within the cell cytosol or if they were secreted additionally into the surrounding milieu in any significant amount. Ensheathing cells were analysed for BDNF, NGF, NT-3 and GDNF using this technique. For each assay of the respective growth factors, a standard curve was generated from which the total concentration of each factor in the test sample was determined. Analysis of ECs gave a total of 182–210 pg/ml of BDNF detected per flask, where each flask contained ~1×10^5 cells (Fig. 3). The amount of NGF immunoreactivity observed in ECs ranged from 1140 to 1440 pg/ml per flask (Fig. 3) which was approximately seven times the proportion of BDNF. GDNF and NT-3 were not detected in the cell lysate. The positive control (brain homogenate) showed consistently the presence of BDNF, NGF, NT-3 and GDNF. No immunoreactivity was detected in the negative control (lysis buffer) for any of the factors analysed.

Serum-free medium conditioned by ~1×10^5 cells for a period of 3 days was analysed to determine if ECs secreted each of these factors. Medium conditioned by ECs contained 150–182 pg/ml BDNF and 1350–1410 pg/ml NGF (Fig. 3). GDNF and NT-3 were not detected in conditioned

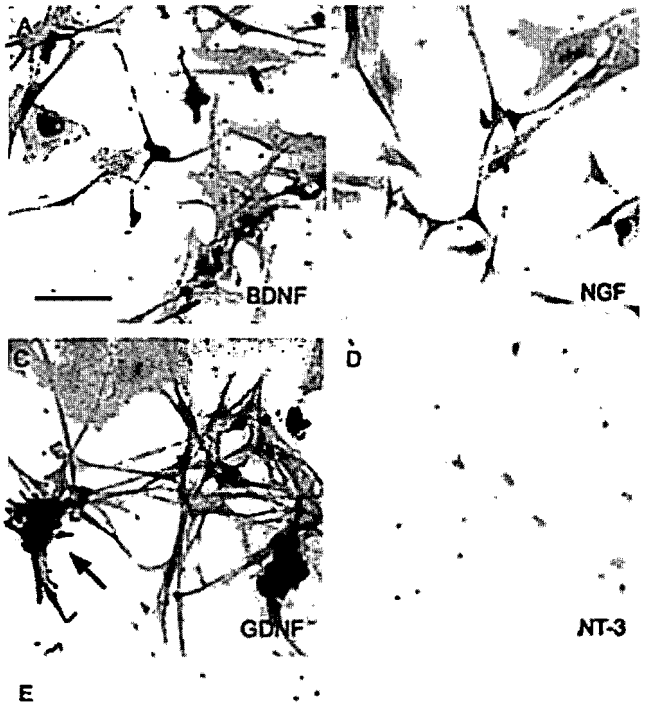

Fig. 2. BDNF (A), NGF (B) and GDNF (C) expression in ensheathing cells as demonstrated by immunohistochemistry. There was variability in staining among cells with the highest intensity being frequently localised to round cells present in clusters (arrow in C). No immunoreactivity for NT-3 was observed (D) and control coverslips with the primary antibody omitted (E) also did not show any staining. Scale bar: 40 μm.

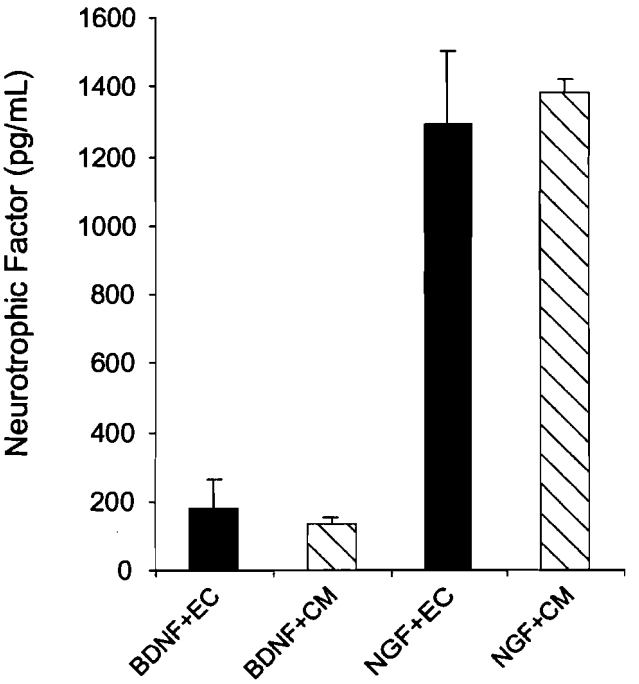

Fig. 3. Quantification of NGF and BDNF present in and secreted by ensheathing cells. ELISA was performed on both ensheathing cell lysate (EC) and medium conditioned by the cells (CM). The results showed that approximately seven times more NGF than BDNF was present in and secreted by ensheathing cells. The error bars indicate standard deviation and each average value was obtained from three separate assays. For each assay, one flask containing approximately 1×10^5 cells were used.

medium. Control (unconditioned) medium contained no detectable presence of any of these factors. These results suggest that ECs contain and secrete BDNF and NGF, however the ELISA may not be sufficiently sensitive to detect the small amount of GDNF present in ECs.

3.3. Ensheathing cells express NGF, BDNF, GDNF and NTN messenger RNA

To confirm the results of the immunohistochemical staining and ELISA, RT-PCR analysis was performed to demonstrate the presence of mRNA for BDNF, NGF, NT-3 and GDNF in ECs. Analysis of olfactory ECs revealed BDNF transcripts of the correct size containing exons 1, 3 and 4 (Fig. 4). Control tissue, fibroblasts gave similar results while the OB expressed only two exons. The distribution of BDNF transcripts in these tissues is consistent with the finding that expression of BDNF mRNA is tissue specific due to the different promoter regions within the gene [40].

Analysis of NGF in ECs produced two fragments at 300 and 500 base pairs, respectively (Fig. 5). The larger product was closer to the expected size (Table 1) while the shorter transcript may represent a different alternatively spliced isoform of NGF. Olfactory bulb and brain expressed only the 300 base pair fragment which is reportedly most abundant in peripheral tissues and brain regions [54]. Two transcripts corresponding to 300 and 500 base pairs were also detected in fibroblasts.

It was concluded that NT-3 mRNA was absent from ECs as no bands were observed for NT-3 1A or NT-3 1B (Fig. 6). The NT-3 1B fragment was detected in both brain

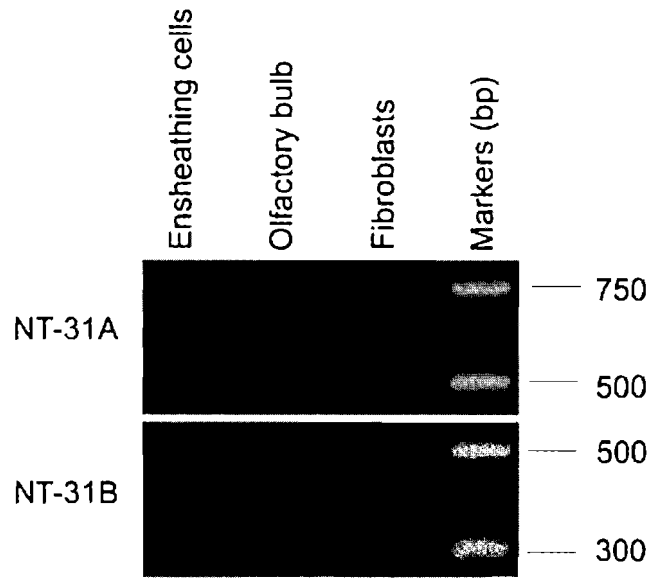

Fig. 5. RT-PCR analysis of NT-3 isoforms in ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells failed to express NT-3 1A and 1B while the olfactory bulb was shown to express NT-3 1B.

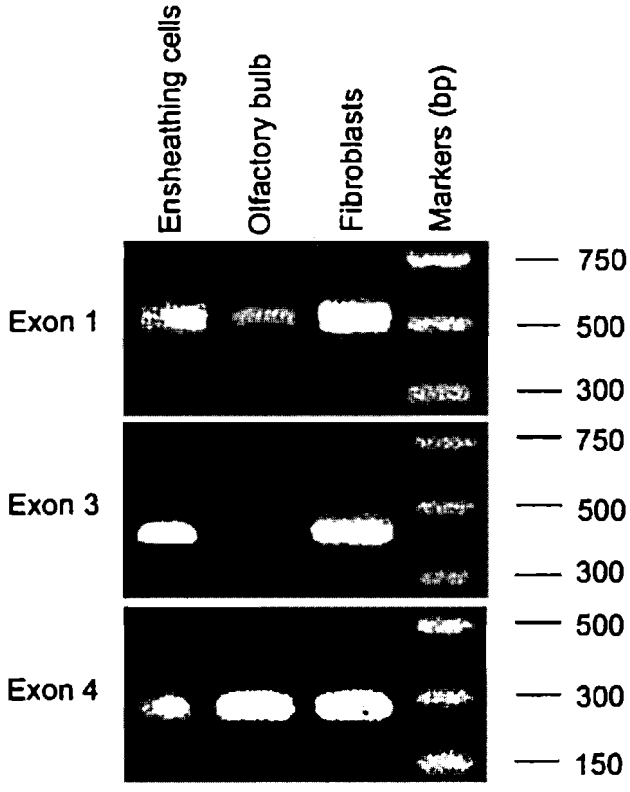

Fig. 4. RT-PCR analysis of alternatively spliced BDNF isoforms in rat ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells produced bands of the appropriate size for BDNF transcripts corresponding to exons 1, 3 and 4. The olfactory bulb did not express exon 3 while all three exon fragments were found in fibroblasts.

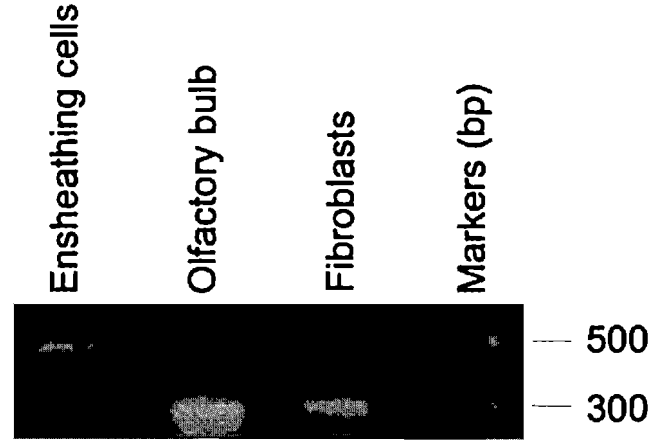

Fig. 6. RT-PCR analysis of NGF in rat ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells expressed two fragments corresponding to 300 and 500 base pairs while the olfactory bulb only showed the 300 base pair fragment.

(data not shown) and OB while no transcripts were found in fibroblasts. These results indicate again that NT-3 mRNA isoforms are differentially expressed in different tissues.

A single GDNF mRNA (Table 1) corresponding to ~660 base pairs was detected in ECs while the olfactory bulb and fibroblasts were shown to possess two separate mRNA products, at ~660 and ~590 base pairs (Fig. 7). In an earlier study by Suter-Crazzolara and Unsicker [59] two similar bands were produced using the same primer set. These transcripts were termed lGDNF and sGDNF for the large (660 bp) and small (590 bp) fragments, respectively [58]. The shorter transcript is thought to arise by alternative splicing and contains a sequence deletion [58].

NTN mRNA of the correct size (483 bp) was expressed in ECs (Fig. 8). A similar band was also detected in OB while fibroblasts appeared to express an additional band corresponding roughly to 740 base pairs.

3.4. Olfactory ensheathing cells express neurotrophin and GDNF receptor genes

To determine whether ECs produce mRNA for the neurotrophin receptors trkA, trkB and trkC and the GDNF family receptors GFRα-1, GFRα-2 and RET, RT-PCR analysis was performed. The results showed that ECs produced an mRNA fragment at the expected size of trkB, but no transcripts for trkA or trkC were detected (Fig. 9). The fibroblast and OB controls expressed single transcripts for both trkB and trkC. The trkA transcript was not detected in any of the tissues analyzed.

RT-PCR analysis showed that ECs expressed mRNA for the GFRα-1 and GFRα-2 receptors but not for the signal transducing receptor RET (Fig. 10). The control tissues,

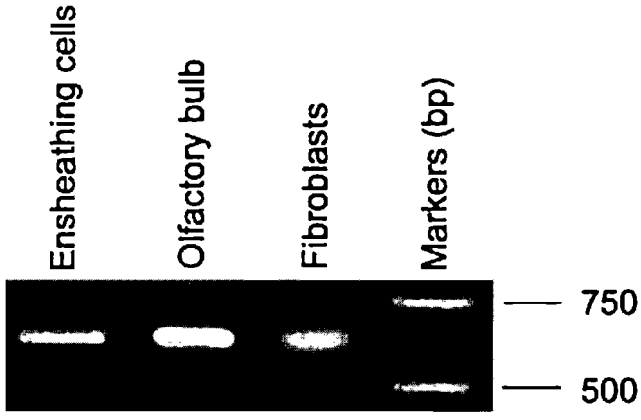

Fig. 7. RT-PCR analysis of GDNF in ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells possessed transcripts corresponding only to lGDNF (660 base pairs) while the olfactory bulb and fibroblasts expressed both lGDNF and sGDNF (590 base pairs).

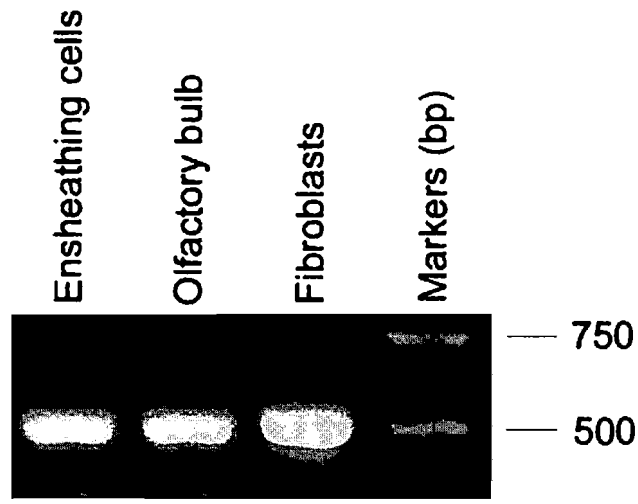

Fig. 8. RT-PCR analysis of NTN in ensheathing cells, olfactory bulb and fibroblasts. All three tissues expressed NTN corresponding to a fragment of 483 base pairs. Another band representing a larger fragment was seen in fibroblasts.

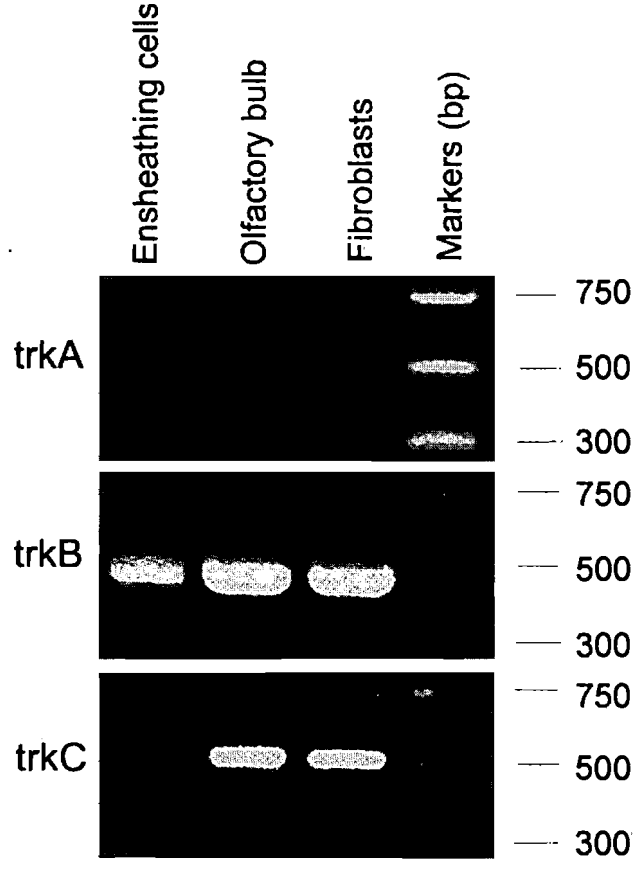

Fig. 9. RT-PCR analysis of neurotrophin receptors in ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells expressed trk B but not trk A and C. Both trk B and C were present in the olfactory bulb and fibroblasts.

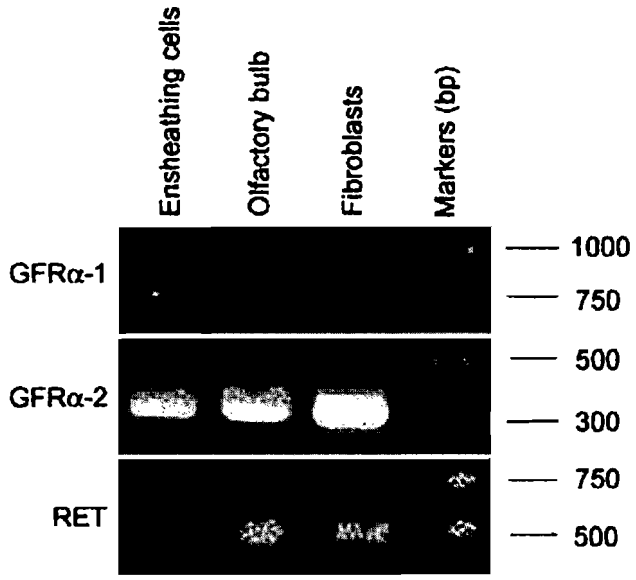

Fig. 10. RT-PCR analysis of GDNF family receptors in ensheathing cells, olfactory bulb and fibroblasts. Ensheathing cells expressed GFRα-1 and GFRα-2 but not RET. All three GDNF receptors were present in olfactory bulb and fibroblasts.

olfactory bulb and fibroblasts expressed GFRα-1, GFRα-2 and RET.

4. Discussion

Results of this project provide the first direct evidence that cultured ECs produce a number of growth factors. Ensheathing cells express the messenger RNA and protein for NGF, BDNF and GDNF. Both NGF and BDNF are secreted while secretion of GDNF was not detected by ELISA. Ensheathing cells did not appear to express NT-3. In addition RT-PCR demonstrated that ECs also express NTN and growth factor receptors Trk B, GFRα-1 and GFRα-2.

This study has shown clearly that ECs synthesize and secrete NGF and BDNF protein. Although there have been no reports of NGF or BDNF in ECs, previous studies have localised NGF to the dendrites and axons of olfactory neurons [1,62] and BDNF to horizontal basal cells in the olfactory neuroepithelium [8]. The demonstration of NGF mRNA in the OB in this study is consistent with earlier observations that periglomerular cells, as well as the internal and external plexiform layers of the OB show immunoreactivity for NGF [55]. The periglomerular and granule cell layers of the olfactory bulb have also been shown to be positive for BDNF mRNA [14,26].

The precise function of NGF and BDNF in the olfactory pathway has not been defined clearly although a number of in vitro studies have attempted to elucidate their role. In one study it was shown that NGF increased olfactory neuronal survival and neurite extension [50] but in later reports, it was demonstrated that NGF failed to promote cell division as measured by radiolabeled DNA precursor [18,27]. It has been shown that NGF, BDNF and NT-3 act in concert to induce c-Fos expression and to increase the number of differentiating olfactory neurons [51]. In another study it was shown that although BDNF, in the presence of transforming growth factor-β2, enhanced initial plating efficiency of olfactory neurons, it could not sustain survival beyond 10 days [35]. Most recently NGF was found to enhance proliferation and survival of immortalized olfactory cells generated from a transgenic mouse while BDNF and NT-3 promoted their differentiation [6]. Hence results from in vitro studies are variable and point to the likelihood that olfactory neuronal differentiation and survival are probably regulated by complex interaction with different types of growth factors or molecules.

There have also been experiments that point to a role for BDNF in the OB. When BDNF was infused unilaterally into the lateral ventricles, the number of proliferating cells, as indicated by bromodeoxyuridine uptake, increased in all layers of the ipsilateral olfactory bulb [63]. These dividing cells were thought to be interneurons arising in the subventricular zone and arriving in the OB by way of the rostral migratory stream [38]. In view of this finding, it can be suggested that BDNF promotes proliferation of olfactory bulb interneurons. Alternatively, the newly generated cells could represent the surviving neurons in the subventricular zone, many of which would have died under normal conditions before they migrate to the OB. Ensheathing cells are not the only source of BDNF in the olfactory bulb; earlier studies have revealed that periglomerular and granule cells also possess BDNF mRNA [14,26]. Additionally our findings show that ECs expressed mRNA for trkB the receptor for BDNF. The presence of this receptor suggests that BDNF has an autocrine effect on ECs. Further investigation will be needed to determine whether BDNF is involved in the activation of the MAPK pathway thereby eliciting changes in the nuclear activity of ECs.

Results of RT-PCR analysis in this study demonstrate that growth factor expression in cultured ECs and the olfactory bulb is not totally congruent. The olfactory bulbs used in the RT-PCR had an intact olfactory nerve layer and thus contained a significant population of ECs. The results showed that ECs in culture expressed isoforms of NGF and BDNF mRNA which were not found in the olfactory bulb, i.e. exon 3 of BDNF and the 500 bp NGF isoform (Figs. 4 and 5). This indicates that the specific culture conditions could be responsible for inducing expression of these neurotrophin isoforms. On the other hand, they failed to express NT-3 1B which was detected in the olfactory bulb (Fig. 6) suggesting that some form of inhibitory regulation may be acting in the cultures. Conversely, because ECs represent only one of several cell types in the olfactory bulb, the latter may be exerting a stimulatory effect on the

expression of NT-3 in situ or alternatively NT-3 expression in the bulb is attributed to the other cell types. The presence of NT-3 mRNA has been a point of contention. Some earlier studies have failed to locate NT-3 mRNA in the olfactory bulb [14,26] yet others have detected NT-3 protein by ELISA [29]. Taken together with the results of this study, it is clear that NT-3 is indeed expressed in the olfactory bulb. It would be worthwhile in future studies to characterize the specific cell types that are responsible for the synthesis of this protein in the OB in vivo.

Immunohistochemical analysis and RT-PCR indicated that ECs express GDNF. Despite this no GDNF protein was detected by the ELISA in ECs or in medium conditioned by ECs. It seems likely then that this is due to the fact that ensheathing cells synthesize and possibly secrete very small quantities of the protein and that this is below the detection limit of the ELISA. Currently it is not possible to use a bioassay to detect the secretion of GDNF because the amount produced is below 30 pg/ml which is about 1000-fold less than that required for a biological effect [36].

In our study, GDNF mRNA was also detected in the olfactory bulb, consistent with a previous study which, using in situ hybridization, localised GDNF mRNA to the granular layer of the OB [60]. Interestingly, immunohistochemical staining for GDNF protein showed a more extensive distribution being present in the olfactory nerve layer, mitral cells and tufted cells scattered in the external plexiform layer, and to a lesser degree some periglomerular and granule cells [8]. However there has been no mention of immunoreactivity in the ensheathing cells residing in the olfactory nerve layer. This apparent discrepancy with our cell culture results suggests that regulatory mechanisms suppressing GDNF synthesis may be operating in vivo and that future investigation into intercellular interaction could shed some light on this subject.

Our study show that ECs expressed mRNA for GFRα-1 and GFRα-2, the receptors for GDNF and NTN, respectively, but not RET. Although GFRα confer ligand specificity, they lack the capacity for signal transduction that is normally provided by RET [2,49]. Previous studies have localized GFRα to the olfactory epithelium [42] and extensively in the bulb including the glomerular, external plexiform, mitral and granular layers [21,60]. RET, however, is predominantly present in the glomerular layer [21,60]. Since ECs lacked RET expression it is unlikely that GDNF and NTN could elicit intracellular cytoplasmic signals within these cells. It is possible that the respective receptors on ECs function to bind and present GDNF and NTN to growing neurons, resembling the situation observed in lesioned sciatic nerve [60]. In our study NTN mRNA was detected in the OB despite the fact that an earlier study by Golden et al. [21] using in situ hybridization failed to locate it in the same tissue. There have been no direct reports regarding the roles of GDNF and NTN in the OB, hence their function remains unclear.

In summary we have clearly shown that cultured ECs express NGF, BDNF, GDNF, NTN and their receptors. These findings build on earlier studies that demonstrate that ECs contain neuregulins including GGF2 [13,52]. Taken together, the data suggest that ECs are a rich source of growth factors. These findings have important implication for the role of ECs in olfactory neuron differentiation as well as their use in stimulating axonal regeneration in the injured spinal cord. Previous tissue culture studies revealed that medium conditioned by ECs failed to promote neurite growth from olfactory [11] and retinal ganglion neurons [57]. This led to the conclusion that ECs probably mediate neurite outgrowth by direct membrane contact. However, a recent study by Kafitz and Greer [28] revealed that if olfactory neurons and ECs were spatially separated but growing together in the same petri dish, the length of the neurites was similar to that obtained when olfactory neurons were cultured in direct contact with ECs. These findings indicate the involvement of diffusible factors from ECs in promoting neurite growth and also that these diffusible factors may be inactivated rapidly or required in high quantities by the differentiating neurons. Given that this study has identified and quantified some of the growth factors produced by ECs, further investigation can now be carried out to define more precisely the role of ECs in mediating olfactory nerve development.

Just as important, the results of this study have given new insight into the possible mechanism by which ECs are able to stimulate regeneration in the injured CNS. By identifying the growth factors secreted by ECs, future experiments will be able to utilize ECs more efficiently to meet the cellular and trophic requirements for regeneration in the CNS. An option is to augment EC injections into the injured spinal cord with growth factors such as NGF and BDNF.

Acknowledgements

This work was funded by an Australian Research Council grant and the Motor Accident Insurance Board (Tasmania). We thank J. Harris and B. Eckhardt for technical and photographic assistance, respectively.

References

[1] T. Aiba, J. Mori, Y. Nakai, Nerve growth factor (NGF) and its receptor in rat olfactory epithelium, Acta Otolarygol. (Stockh.) Suppl. 506 (1993) 37–40.

[2] M. Airaksinen, A. Titevsky, M. Saarma, GDNF family neurotrophic factor signaling: four masters, one servant?, Mol. Cell Neurosci. 13 (1999) 313–325.

[3] L. Astic, V. Pellier-Monnin, F. Godinot, Spatio-temporal patterns of ensheathing cell differentiation in the rat olfactory system during development, Neuroscience 84 (1998) 295–307.

[4] M. Barbacid, The trk family of neurotrophin receptors, J. Neurobiol. 251 (1984) 386–403.
[5] P.C. Barber, R.M. Lindsay, Schwann cells of the olfactory nerves contain glial fibrillary acidic protein and resemble astrocytes, Neuroscience 7 (1982) 3077–3090.
[6] R.D. Barber, D.E. Jaworsky, K.-W. Yau, G.V. Ronnett, Isolation and in vitro differentiation of conditionally immortalized murine olfactory receptor neurons, J. Neurosci. 20 (2000) 2695–3704.
[7] J.E. Bottenstein, G.H. Sato, Growth of rat neuroblastoma cell line in serum-free supplemented medium, Proc. Natl. Acad. Sci. USA 76 (1979) 514–517.
[8] M.E. Buckland, A.M. Cunningham, Alterations in expression of the neurotrophic factors glial cell line-derived neurotrophic factor, ciliary neurotrophic factor and brain-derived neurotrophic factor, in the target-deprived olfactory neuroepithelium, Neuroscience 90 (1999) 333–347.
[9] P. Chromczynski, N. Sacchi, Single step method of RNA isolation by guanidium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[10] M.I. Chuah, C. Au, Cultures of ensheathing cells from neonatal rat olfactory bulbs, Brain Res. 601 (1993) 213–220.
[11] M.I. Chuah, C. Au, Olfactory cell cultures on ensheathing cell monolayers, Chem. Senses 19 (1994) 25–34.
[12] M.I. Chuah, R. Teague, Basic fibroblast growth factor in the primary olfactory pathway: mitogenic effect on ensheathing cells, Neuroscience 88 (1999) 1043–1050.
[13] M.I. Chuah, J.-M. Cossins, E. Woodhall, R. Tennent, G. Nash, A.K. West, Glial growth factor 2 induces proliferation and structural changes in ensheathing cells, Brain Res. 857 (2000) 265–274.
[14] M.L. Deckner, N.J. Fris, V.M. Verge, T. Hokfelt, M. Risling, Localisation of neurotrophin receptors in olfactory epithelium and bulb, NeuroReport 5 (1993) 301–304.
[15] J.R. Doucette, Glial influences on axonal growth in the primary olfactory system, Glia 3 (1990) 433–449.
[16] J.R. Doucette, J.A. Kiernan, B.A. Flummerfelt, The re-innervation of olfactory glomeruli following transection of primary olfactory axons in the central or peripheral nervous system, J. Anat. 137 (1983) 1–19.
[17] R. Doucette, Glial cells in the nerve fiber layer of the main olfactory bulb of embryonic and adult mammals, Microsc. Res. Tech. 24 (1993) 113–130.
[18] A.I. Farbman, J.A. Buchholz, Transforming growth factor-alpha and other growth factors stimulate cell division in olfactory epithelium in vitro, J. Neurobiol. 30 (1996) 267–280.
[19] A.I. Farbman, Cell Biology of Olfaction, Cambridge University Press, Cambridge, 1992, pp. 55–62.
[20] I. Franceschini, S. Barnett, Low-affinity NGF-receptor and EN-CAM expression define two types of olfactory nerve ensheathing cells that share a common lineage, Dev. Biol. 289 (1996) 481–492.
[21] J.P. Golden, R.H. Baloh, P.T. Kotzbauer, P.A. Lamp, P.A. Osborne, J. Milbrandt, E.M. Johnson, Expression of neurturin, GDNF and their receptors in the adult mouse CNS, J. Comp. Neurol. 398 (1998) 139–150.
[22] Q. Gong, M. Shipley, Expression of extracellular matrix molecules and cell surface molecules in the olfactory nerve pathway during early development, J. Comp. Neurol. 366 (1996) 1–14.
[23] Q. Gong, M.S. Bailey, S. Pixley, M. Ennis, W. Liu, M.T. Shipley, Localisation and regulation of low affinity nerve growth factor receptor expression in the rat olfactory system during development and regeneration, J. Comp. Neurol. 344 (1994) 336–348.
[24] P.P.C. Graziadei, G.A. Monti-Graziadei, Neurogenesis and neuron regeneration in the olfactory system of mammals. III. Deafferentation and reinnervation of the olfactory bulb following section of the fila olfactoria in rat, J. Neurocytol. 9 (1980) 145–162.
[25] P.P.C. Graziadei, G.A. Monti Graziadei, Continuous nerve cell renewal in the olfactory system, in: M. Jacobson (Ed.), Handbook of Sensory Physiology, Vol. IX, Springer, Berlin, 1978, pp. 55–82.
[26] K.M. Guthrie, C.M. Gall, Differential expression of mRNAs for the NGF family of neurotrophic factors in the adult rat central olfactory system, J. Comp. Neurol. 313 (1991) 95–102.
[27] J. Holcomb, J.S. Mumm, A.L. Calof, Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro, Dev. Biol. 172 (1995) 307–323.
[28] K.W. Kafitz, C.A. Greer, Olfactory ensheathing cells promote neurite extension from embryonic olfactory receptor cells in vitro, Glia 25 (1999) 99–110.
[29] R.R.S. Katoh-Semba, I. Takeuchi, R. Kato, Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3, Neurosci. Res. 31 (1998) 227–234.
[30] P.T. Kotzbauer, P.A. Lampe, R.O. Heuckeroth, J.P. Golden, D.J. Creedon, E.M. Johnson Jr., J. Milbrandt, Neurturin, a relative of glial-cell-line-derived neurotrophic factor, Nature 384 (1996) 467–470.
[31] Y. Li, P.M. Field, G. Raisman, Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells, Science 277 (1997) 2000–2002.
[32] T. Lenhard, C. Suter-Crazzolara, Developmental expression of GDNF, neurturin and their receptors in rat hippocampus, Neuroreport 9 (1998) 2927–2931.
[33] P. Liesi, Laminin-immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones, EMBO J. 4 (1985) 2505–2511.
[34] A. Mackay-Sim, P.W. Kittel, On the life span of olfactory receptor neurons, Eur. J. Neurosci. 3 (1991) 209–215.
[35] N.K. Mahanthappa, G.A. Schwarting, Peptide growth factor control of olfactory neurogenesis and neuron survival in vitro: roles of EGF and TGF-βs, Neuron 10 (1993) 293–305.
[36] R.E. McAlhany Jr., J.R. West, R.C. Miranda, Glial-derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JUN kinase phosphorylation, Dev. Brain Res. 119 (2000) 209–216.
[37] S.O. Meakin, E.M. Shooter, The nerve growth factor family, Trends Neurosci. 15 (1992) 323–331.
[38] J. Menezes, C. Smith, K. Nelson, M. Luskin, The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain, Mol. Cell Neurosci. 6 (1995) 496–508.
[39] X. Navarro, A. Valero, G. Gudino, J. Fores, F.J. Rodriguez, E. Verdu, R. Pascual, J. Cuadras, M. Nieto-Sampedro, Ensheathing glia transplants promote dorsal root regeneration and spinal reflex restitution after multiple lumbar rhizotomy, Ann. Neurol. 45 (1999) 207–215.
[40] K. Nemoto, K. Fukamachi, F. Nemoto, S. Miyata, M. Hamada, Y. Nakamura, E. Senba, T. Ueyama, Gene expression of the neurotrophins and their receptors in cultured rat vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 194 (1998) 231–238.
[41] R.B. Norgren, N. Ratner, R. Brackenbury, Development of olfactory nerve glia defined by a monoclonal antibody specific for Schwann cells, Dev. Dyn. 194 (1992) 231–238.
[42] C.A. Nosrat, A. Tomac, B.J. Hoffer, L. Olson, Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor α mRNAs, Exp. Brain Res. 115 (1997) 410–422.
[43] A.J. Okragly, M. Haak-Frenscho, An acid-treatment method for the enhanced detection of GDNF in biological samples, Exp. Neurol. 145 (1997) 592–596.
[44] S. Pixley, The olfactory nerve contains two populations of glia, identified both in vivo and in vitro, Glia 5 (1992) 269–284.
[45] G. Raisman, Specialised neuroglial arrangement may explain the capacity of vomeronasal axons to re-innervate central neurons, Neuroscience 14 (1985) 237–254.
[46] A. Ramon-Cueto, M. Nieto-Sampedro, Regeneration into the spinal cord of transected dorsal root axons is promoted by ensheathing glia transplants, Exp. Neurol. 127 (1994) 232–244.

[47] A. Ramon-Cueto, M. Nieto-Sampedro, Glial cells from adult rat olfactory bulb: immunocytochemical properties of pure cultures of ensheathing cells, Neuroscience 47 (1992) 213–220.
[48] A. Ramon-Cueto, G.W. Plant, J. Avila, M. Bunge, Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia, J. Neurosci. 18 (1998) 3808–3815.
[49] K. Robertson, I. Mason, The GDNF-RET signalling partnership, Trends Genet. 13 (1997) 1–3.
[50] G.V. Ronnett, L.D. Hester, S.H. Snyder, Primary culture of neonatal rat olfactory neurons, J. Neurosci. 11 (1991) 1243–1255.
[51] A.J.I. Roskams, M.A. Bethel, K.J. Hurt, G.V. Ronnett, Sequential expression of trks A, B and C in the regenerating olfactory neuroepithelium, J. Neurosci. 16 (1996) 1294–1307.
[52] K. Salehi-Ashtiani, A.I. Farbman, Expression of *neu* and Neu differentiation factor in the olfactory mucosa of rat, Int. J. Dev. Neurosci. 14 (1996) 801–811.
[53] B. Scheffler, M. Horn, I. Blumcke, E. Laywell, D. Coome, V. Kukekov, D. Steindler, Marrow-mindedness: a perspective on neuropoiesis, Trends Neurosci. 22 (1999) 348–357.
[54] M.J. Selby, R. Edwards, F. Sharp, W.J. Rutter, Mouse nerve growth factor gene: structure and expression, Mol. Cell Biol. 7 (1987) 3057–3064.
[55] M.-C. Senut, Y. Lamour, J. Lee, P. Brachet, E. Dicou, Neuronal localisation of the nerve growth factor precursor-like immunoreactivity in the rat brain, Int. J. Dev. Neurosci. 8 (1990) 65–80.
[56] K.A. Smale, R. Doucette, M.D. Kawaja, Implantation of olfactory ensheathing cells in the adult rat brain following fimbria-fornix transection, Exp. Neurol. 137 (1996) 225–233.
[57] R.J. Sonigra, P.C. Brighton, J. Jocoby, S. Hall, C.B. Wigley, Adult rat olfactory nerve ensheathing cells are effective promoters of adult central nervous system neurite outgrowth in coculture, Glia 25 (1999) 256–269.
[58] C. Suter-Crazzolara, K. Unsicker, GDNF is expressed in two forms in many tissues outside the CNS, NeuroReport 5 (1994) 2486–2488.
[59] C. Suter-Crazzolara, K. Unsicker, GDNF mRNA levels are induced by FGF-2 in rat C-6 glioblastoma cells, Mol. Brain Res. 41 (1996) 175–182.
[60] M. Trupp, N. Belluardo, H. Funakoshi, C.F. Ibanez, Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-α indicates multiple mechanisms of trophic actions in the adult rat CNS, J. Neurosci. 17 (1997) 3554–3567.
[61] H. Vickland, L.E. Westrum, J.N. Kott, S.L. Patterson, M.A. Bothwell, Nerve growth factor receptor expression in the young and adult rat olfactory system, Brain Res. 565 (1991) 269–279.
[62] R. Williams, R.A. Rush, Electron microscopic immunocytochemical localization of nerve growth factor in developing mouse olfactory neurons, Brain Res. 463 (1988) 21–27.
[63] T. Zigova, V. Pencea, S. Wiegand, M. Luskin, Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb, Mol. Cell Neurosci. 11 (1998) 234–245.