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Abstract

Neuroblasts born in the subventricular zone (SVZ) migrate along the rostral migratory stream, reaching the olfactory bulb (OB) where they differentiate into local interneurons. Several extracellular factors have been suggested to control specific steps of this process. The brain-derived neurotrophic factor (BDNF) has been demonstrated to promote morphological differentiation and survival of OB interneurons. Here we show that BDNF and its receptor TrkB are expressed in vivo throughout the migratory pathway, implying that BDNF might also mediate migratory signals. By using in vitro models we demonstrate that BDNF promotes migration of SVZ neuroblasts, acting both as inducer and attractant through TrkB activation. We show that BDNF induces cAMP response element- binding protein (CREB) activation in migrating neuroblasts via phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAP-K) signalling. Pharmacological blockade of these pathways on SVZ explants significantly reduces CREB activation and impairs neuronal migration. This study identifies a function of BDNF in the SVZ system, which involves multiple protein kinase pathways leading to neuroblast migration.

Introduction

In adult rodents, olfactory bulb (OB) interneurons are continuously generated from precursors cells of the subventricular zone (SVZ), a remnant of the primitive germinal zones of the embryonic lateral ganglionic eminence (Altman, 1969; Luskin, 1993; Lois & Alvarez-Buylla, 1994; Wichterle et al., 2001). Adult OB neurogenesis is characterized by a unique process of tangential migration from the wall of the lateral ventricle (LV) to the OB along the rostral migratory stream (RMS), a well-defined route, where migrating cells form chains ensheathed by a meshwork of astrocytes, the glial tubes (Lois et al., 1996; Peretto et al., 1997). Upon reaching the OB core, single neuroblasts spread radially migrating towards the granule and glomerular layers, where they undergo differentiation and functional integration (Petreanu & Alvarez-Buylla, 2002; Belluzzi et al., 2003; Carleton et al., 2003). Multiple factors, including cell adhesion molecules, such as polysialic acid neural cell adhesion molecule (PSA-NCAM; Tomasiewicz et al., 1993; Ono et al., 1994; Hu et al., 1996), extracellular matrix molecules (i.e. tenascin-C; Jankovski & Sotelo, 1996; Murase & Horwitz, 2002), and members of the ErbB and Eph family of tyrosine kinase receptors and their ligands (Conover et al., 2000; Anton et al., 2004), have been demonstrated to be involved in neuroblast tangential migration. Directional migration toward the OB is regulated by cooperation of chemorepulsive mechanisms through Slit-Robo signalling (Wu et al., 1999) and chemoattractive cues produced by the OB (Liu & Rao, 2003), such as the secreted molecules netrin-1, prokineticin2 and GDNF (Srinivasan et al., 1998; Murase & Horwitz, 2002; Paratcha et al., 2006). In addition, reelin and tenascin-R have been described to control neuroblast detachment from the chains and

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radial migration in the OB (Hack et al., 2002; Saghatelyan et al., 2004). Different aspects of the migratory process are controlled by various factors, and brain-derived neurotrophic factor (BDNF) appears as another promising candidate. BDNF and its high-affinity receptor TrkB have been previously reported to be expressed in the adult OB (Mackay-Sima & Chuahb, 2000). Moreover, TrkB receptor expression by SVZ-derived neuronal precursors has been demonstrated both in vivo and in vitro (Zigova et al., 1998; Gascon et al., 2005). Although the function of BDNF in this system has so far been correlated with differentiation and survival of the newly generated cells (Ahmed et al., 1995; Kirschenbaum & Goldman, 1995; Pincus et al., 1998; Zigova et al., 1998), ongoing findings on the role exerted by BDNF in the control of cortical and cerebellar interneurons migration during development (Behar et al., 1997; Borghesani et al., 2002; Ohmiya et al., 2002; Polleux et al., 2002; Alcantara et al., 2006) drove us to investigate its involvement in the regulation of SVZ-neuroblast migration. Here we show that BDNF and its high-affinity receptor TrkB are expressed along the migratory pathway. We also provide evidence that BDNF acting on TrkB receptors stimulates cAMP response element-binding protein (CREB) activation and migration of SVZ precursor cells in vitro via phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAP-K) signalling pathways. Besides playing an inductive role on neuronal migration, our results also demonstrate that BDNF is a chemotropic factor, contributing to the directional migration of SVZ neuroblasts.

Materials and methods

Animals

Experiments were performed on newborn [postnatal day 4 (P4)–P10] and young adult (1–2 months old) CD1 strain mice (Charles River, Calco, Italy), kept four-to-five per cage in rooms with a lighting schedule of 12 h light : darkness, and with standard diet and water ad libitum. For all experiments adult animals were deeply anaesthetized by means of an intraperitoneal injection of a ketamine solution (100 mg/kg body weight; Ketavet, Farmaceutici Gellini, Italy) and Òxylazine (33 mg/kg body weight; Rompun , Bayer, Germany). Postnatal mice were anaesthetized by hypothermia. All experimental procedures were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), the Italian law for care and use of experimental animals (DL116/92), and approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin.

Reverse transcriptase-real-time polymerase chainre action (RT-real-time PCR)

Microdissections of SVZ-LV and SVZ-OB tissues were performed on fresh 300-μm vibratome slices under a high-magnification dissecting stereomicroscope (MZ6, Leica, Germany). Dissection of the SVZ-LV from the adjacent striatum could result in a small amount of striatal tissue in the SVZ-LV samples. Thus, as a control we dissect small portions of the striatum and run the analysis in parallel on these samples. Specimens from six adult mice were pooled, collected in TRIzol (Invitrogen, California, USA) and stored at -80 °C until use for RNA and protein extraction. Total RNAs were extracted with TRIzol (Invitrogen), and genomic DNA was removed after DNAse I digestion (RNAse Free DNAse Set, Qiagen, Germany). To facilitate the precipitation of total RNAs from SVZ microdissected samples, glycogen (Ambion, California, USA) was added as a carrier with the TRIzol reagent. After column purification (RNeasy kit, Qiagen) and prior to RT, total RNA from all samples was shown to be free from genomic DNA contamination by a PCR amplification of the exon V of the gene encoding BDNF (see below for details). Messenger RNAs, contained in 500 ng of SVZ total RNAs, were then reverse transcribed with the RT RNase H minus (Promega, Wisconsin, USA) using oligod(T)15, in the presence of 80 pg of a synthetic external and non-homologous poly(A) Standard RNA (SmRNA), used to normalize the reverse transcription of

mRNAs of biological samples (Morales and Bezin, patent WO2004.092414). cDNAs obtained from the reverse transcription of targeted mRNAs were quantified by real-time PCR Òperformed on the LightCycler® System (Roche Diagnostics, Switzer-land) using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Results obtained for the targeted mRNAs were normal-ized against the SmRNA and expressed as number of cDNA copies detected for ng of total RNAs. Sequences of the different primer pairs used are: BDNF(exV) (GeneBank accession no. X67108) forward 5'-AAA TTA CCT GGA TGC CGC AA-3'¢, reverse 5'-CGC CAG CCA ATT CTC TTT TT-3' (345 bp); TrkB (GeneBank accession no. M55291) forward 5'-TGA AGA CGC TGA AGG ACG CCA-3', reverse 5'-CAG GTT CTC TCC TAC CAA GCA-3' (353 bp). All primer pairs were designed using 'Primer 3' software (NIH; http://www.basic.nwu.edu).

Enzyme-linked immunosorbent assay (ELISA)

Proteins were extracted with TRIzol (Invitrogen), according to the manufacturer's instruction, from the same samples used for RNA extraction. The protein levels of BDNF were measured using the BDNF E_{MAX} immunoassay system (Promega) following the manufacturer's protocol. Briefly, each sample was incubated for 2 h at room temperature with shaking in a 96-well plate previously coated with anti-BDNF monoclonal antibody at 4 °C overnight and blocked for 1 h with Block and Sample 1 · buffer. Wells were washed in Tris-buffered saline Tween-20 (TBST) pH 7.4, and incubated with anti-human BDNF polyclonal antibody on a shaking platform for 2 h at room temperature. After rinsing with TBST, anti-IgY-horseradish peroxidase conjugate was added and incubated on a shaking platform for 1 h at room temperature followed by rinsing with TBST and incubation with tetramethyl benzidine for colour development. After 10 min, the reaction was stopped with 1 N HCl and the plate was read at 450 nm on a microplate reader (Model 550; Bio-Rad Laboratories, Segrate Milano, Italy). The amount of BDNF protein in each well was determined by interpolation to the linear range of a curve obtained in the same assay with known concentrations of recombinant human BDNF (standard curve). Results were expressed as pg of BDNF detected for µg of soluble protein.

Immunohistochemistry

Adult mice were transcardially perfused with ice-cold 0.9% NaCl followed by a freshly prepared solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. After dissection, brains were postfixed overnight in the same fixative, then cryoprotected in ascending sucrose solutions, embedded in TissueTek OCT (optimal cutting compound; Sakura Finetek, CA, USA), frozen in liquid nitrogen-cooled isopentane at -70 °C and cryostat sectioned. Free-floating coronal sections (25 µm) were collected in multiwell dishes in series representing the whole forebrain. Sections were stored at -20 °C in antifreeze solution until use. Double-immunofluorescence reactions were performed on floating sections using primary antibodies directed against TrkB (1⁄200; rabbit anti-TrkB, Chemicon, CA, USA) and glial fibrillary acidic protein (1⁄1000; mouse monoclonal anti-GFAP, Boehringer-Mannheim, IN, USA), or PSA-NCAM (1⁄2500; mouse monoclonal IgM anti-PSA-NCAM, AbCys, France). Sections were rinsed in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) and incubated overnight at 4 °C with primary antibodies diluted in PBS containing 1% non-immune serum of the same donor species of the secondary antibodies. The next day, sections were rinsed in PBS and incubated for 1 h at room temperature with the following secondary antibodies: anti-rabbit CY3 (1⁄800; Jackson ImmunoResearch Laboratories, PA, USA) and anti-mouse biotinylated (1⁄250; Vector Laboratories, CA, USA) followed by streptavidin conjugated to Fluorescein (1⁄300; Jackson ImmunoResearch Laboratories). All incubations were performed on a rotating platform. Sections were coverslipped with a DABCO (Sigma-Aldrich, MO, USA)-based antifade mounting medium.

SVZ explants and cell migration assay

Brains from P4–P10 CD1 mice were removed using an aseptic technique in ice-cold PBS (Gibco ® Invitrogen, CA, USA) with 0.6% glucose. Forebrains were embedded in a solution of 3% low-gelling point agarose (Sigma-Aldrich) in Leibovitz's L-15 medium (Gibco ® Invitrogen), vibratome cut into 250-μm-thick coronal slices and collected in Leibovitz's L-15 medium. Under a high-magnification dissecting microscope (MZ6, Leica), SVZ-rostral extension (SVZ- RE) was identified by its translucent appearance and carefully dissected away from the surrounding brain tissue. SVZ-RE explants were embedded into BD Matrigel ™ growth factor reduced (BD Biosciences, NY, USA) diluted 6/1 with fresh control culture medium consisting of Neurobasal ™ medium (Gibco ® Invitrogen), supplemented with N2 supplement (Gibco ® Invitrogen), 0.5 mm glutamine (Sigma-Aldrich) and 25 ng/mL gentamicine (Seromed, Germany). Explants were plated onto sterile coverslips coated with 10 μg/mL poly-l-lysine hydrobromide (Sigma-Aldrich). In order to test the effect of BDNF on neuronal precursor migration, SVZ-RE explants were treated with culture medium supplemented with BDNF (50 ng/mL, Sigma-Aldrich) or with one of the following inhibitors: TrkB inhibitor K252a (50 nm, Calbiochem, Germany), PI3-K inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (10 μm; LY294002, Sigma-Aldrich) or MAP-K inhibitor 2'-amino-3'- methoxy flavone (10 μm; PD98059, Calbiochem). Treatment was carried out at the same time as plating. When pharmacological inhibitors were used, the final concentration represented at least 1 : 1000 dilution of stock solutions prepared in dimethylsulphoxide (DMSO). Control cultures were treated with medium supplemented with DMSO at the same dilution. The explants were maintained for 1 day in vitro (1 DIV) under 5% CO_2 at 37 °C, fixed with PFA 4% and counterstained by incubation in SYTOX Green nucleic acid stain (20 nm; Molecular Probes ™ Invitrogen, CA, USA) for 1 h at room temperature. Some explants were processed for immunocytochemistry as described in the previous section. The primary antibodies used were: anti-Ki67 (1/2000; mouse monoclonal, ScyTek Laboratories, UT, USA); anti-activated caspase-3 (1/3000; rabbit; CM1 antibody; Idun Pharmaceuticals, CA, USA); anti-pCREB (1/200; rabbit, Cell Signalling, MA, USA); anti-PSA-NCAM (1/2500); and anti-TrkB (1/200).

Chemotaxis assay

The assay was performed using a 48-well Boyden microchemotaxis chamber according to manufacturer's instructions (Neuroprobe, Cabin John, MD, USA). Briefly, SVZ microdissected samples obtained from P4–P10 mice, as previously described, were enzymatically dissociated in a Trypsin-EDTA solution (Sigma-Aldrich). After 5 min at 37 °C, enzymatic dissociation was stopped with heat- inactivated foetal bovine serum (Sigma-Aldrich), followed by mechanical dissociation through a P200 pipette. After centrifugation for 7 min, SVZ cells were collected and resuspended at $1 \cdot 10^6$ cells/mL in control culture medium (see above). Fifty microlitres of 10^6 cells/mL suspension was placed in the open- bottom wells of the upper compartment. Each pair of wells was separated by a poly vinylpyrrolidone-free polycarbonate porous membrane (5-μm pores) precoated with poly-d-lysine hydrobromide (30 μg/mL; Sigma-Aldrich). For chemotaxis (the directed migration of cells toward regions of higher concentration of chemotactic factors) experiments, BDNF (12.5, 25, 50, 100 ng/mL) was placed into the wells of the lower compartment of the chamber. Chemokinesis (stimulation of increased random cell motility) was distinguished from chemotaxis by placing the same concentration of BDNF in both the upper and lower wells of the Boyden chamber, thereby eliminating the chemical gradient. The specificity of the BDNF effect was evaluated using BDNF (50 ng/mL, Sigma- Aldrich) previously adsorbed by an anti-BDNF neutralizing antibody (1/50; Promega) in the lower chamber or by adding K252a inhibitor (50 nm) in both the upper and lower chambers. The Boyden chamber was kept for 24 h in the cell culture

incubator. After incubation, the membrane's upper surface was scraped free of cells and debris, rinsed in PBS, fixed with PFA 4% for 45 min and stained using 4',6-diamidino-2-phenylindole dihydrochloride nucleic acid stain (1⁄500 in PBS; Sigma-Aldrich) for 15 min and mounted onto glass slides. Quantification of cells that had migrated through pores and adhered to the lower surface of the membrane was performed under a fluorescence microscope (1 · 50, Olympus, NY, USA) equipped with a CoolSNAP-Pro colour RS Photometrics camera (Media Cybernetics, MD, USA) by counting cells in four random fields for each well (field's area 1⁄4 1.4 mm ^{2} ; well's area 1⁄4 8 mm ^{2}).

PCREB assay and Western blotting analysis

SVZ-RE explants obtained from P5 mice were incubated for 20 min at 37 °C with control medium (see above), or medium containing BDNF alone (50 ng⁄mL) or BDNF (50 ng⁄mL) in the presence of inhibitors LY 294002 (10 μm) or PD 98059 (10 μm). Proteins from these explants were extracted by boiling in sodium dodecyl sulphate (SDS) 1%. The protein content was determined using the bicinchoninic acid method. Western gels were prepared using 30 lg of proteins released by boiling in sample buffer [62.5 mm Tris (pH 6.8), 4% SDS, 480 mm 2-mercaptoethanol and 40% glycerol] and resolved by 12% SDS– polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a Hybond ^{TM} C Extra membrane (Amersham Biosciences, England) according to the manufacturer's instructions, and blocked overnight at 4 °C with 5% non-fat powder milk in TBST buffer (20 mm Tris, 150 mm NaCl, 0.1% Tween-20, pH 7.4). The membranes were probed with primary antibodies diluted in TBST containing 1% bovine serum albumin (BSA) for 1 h at room temperature, and then incubated with horseradish peroxidase-linked anti-rabbit (1⁄3000, Amersham Bio- sciences) secondary antibody diluted in TBST containing 1% BSA for 1 h at room temperature. Specific binding was detected by the enhanced chemiluminescence (ECL) system (Amersham Biosciences) using Hyperfilm ^{TM} (Amersham Biosciences). Polyclonal antibodies against pCREB (1⁄1000; rabbit, Cell Signalling) and CREB (1⁄1000; rabbit, Cell Signalling) were used for staining. Each membrane was sequentially probed with the two antibodies. After the first antibody incubation and detection, membranes were rinsed with TBST, incubated with the peroxidase-coupled secondary antibody and tested by the ECL detection system to confirm the absence of signals. Only membranes completely free from signals were used for incubation with a second primary antibody. Standardization of protein loading was performed by comparison of the CREB band intensity. Results are expressed as a percentage of control. Statistical comparisons were made on three independent experiments by one-way anova followed by Fisher's LSD post hoc.

Photography and data analysis

All images were captured on a Fluo-View 200 confocal microscope (Olympus Instruments, San Francisco, CA, USA). Confocal image z-stacks were captured through the thickness of the slice⁄explant at 1-μm optical steps. The digital images were brightness, colour and contrast balanced, and assembled into montages using CorelDraw 11 (Corel, Ottawa, Ontario, Canada). Quantification of cell migration from the explants was performed on SYTOX Green-labelled specimens (N 1⁄4 at least 19 explants) The migration area (lm 2) was evaluated on 10 X digitalized images as the surface covered by Sytox Green nuclear staining excluding the tissue explant area. The percentage of cells migrating further than an x distance from the explant border was determined overlaying a calibration grid on 40 ·X multistack images. SYTOX Green-stained nuclei were counted every 50 μm from the tissue border to the migration front. Measures were realized using ImageJ (US National Institutes of Health, MD, USA). Cell proliferation in tissue explants from different experimental groups was evaluated by counting all Ki67-immunopositive cells (N 1⁄4 9 explants), cell survival by counting activated caspase-3-immunopositive cells (N 1⁄4 9 explants). The

percentage of pCREB-positive migrating cells surrounding the explants was evaluated on 50 ·X confocal digitalized images on Sytox Green⁄pCREB double-labelled specimen (N = at least 9 explants).

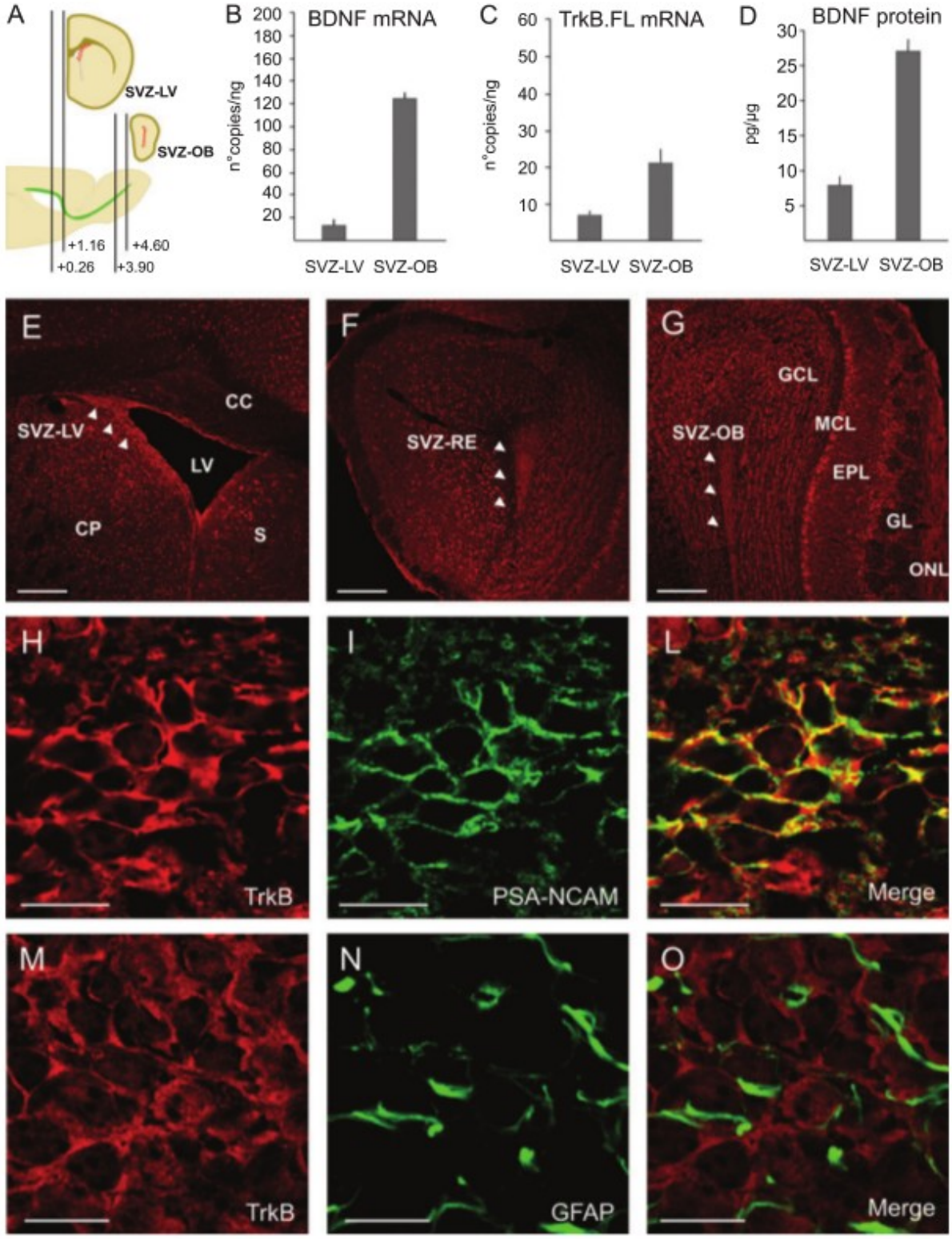

Fig. 1. Brain-derived neurotrophic factor (BDNF) and TrkB expression in the subventricular zone (SVZ) system of the adult mice. (A–D) BDNF and TrkB expression quantified on microdissected SVZ tissues obtained at the level of the lateral ventricle (SVZ-LV; +0.26, +1.16 mm from Bregma) and olfactory bulb (SVZ- OB; +3.90, 4.60 mm from Bregma). Transcripts encoding BDNF and TrkB are detectable both in SVZ-LV and SVZ-OB. However, the expression levels are lower in the SVZ-LV compared with SVZ-OB. BDNF protein expression shows a similar pattern (D). Values are indicated as mean ± standard error of the mean. (E–O) TrkB-immunopositive elements are widely distributed in several regions of the rostral forebrain. Arrowheads indicate TrkB immunostaining in cells of the SVZ-LV (E), SVZ-

rostral extension (SVZ-RE, F) and SVZ-OB (G). TrkB-immunopositive cells in the SVZ are double-labelled for polysialic acid neural cell adhesion molecule (PSA-NCAM; H–L). No co-localization was observed with glial fibrillary acidic protein (GFAP; M–O). Scale bars: 200 lm (E–G); 10 μm (H–O). Abbreviations: CC, corpus callosum; CP, caudate putamen; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; MCL, mitral cell layer; ONL, olfactory nerve layer; S, septum.

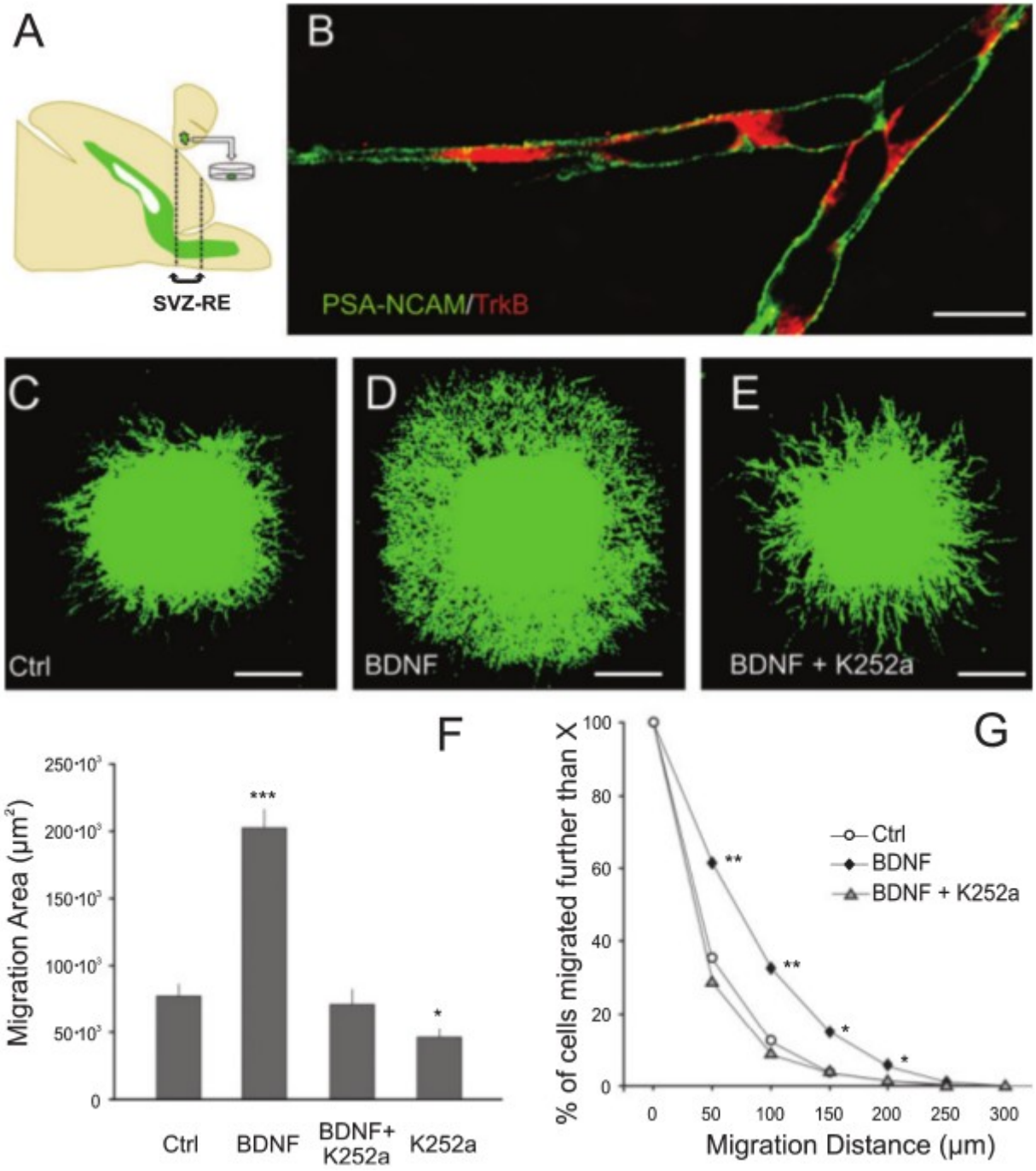

Fig. 2. Brain-derived neurotrophic factor (BDNF) via TrkB receptor enhances migration of neural precursors from subventricular zone (SVZ) explants in vitro. (A) Tissue explants taken from the SVZ-rostral extension (RE) of postnatal mice were cultured in matrigel tridimensional matrix. (B) At 1 DIV, polysialic acid neural cell adhesion molecule (PSA-NCAM)-immunopositive cells migrating out of the explant are double-labelled for TrkB. Scale bar: 10 μm. (C–E) SVZ-RE explants at 1 DIV, stained with SYTOX Green nucleic acid stain. SVZ-RE explants cultured in the presence of BDNF (50 ng⁄mL; D) show an increase in cell migration compared with control (C). The increase in cell migration is blocked by incubating the explants in the presence of TrkB inhibitor K252a (50 nm) (E). Scale bars: 200 μm. (F) Quantification of the surface covered by migrating cells. BDNF treatment produces a marked increase in the migration area (~ 250% of control). K252a inhibitor in the presence of BDNF restores control conditions. K252a alone reduces

the migration area compared with controls. (G) Migration distances covered by cells migrated from SVZ-RE at 1 DIV. Values are expressed as mean ± standard error of the mean. One-way anova followed by Fisher's LSD post hoc was used to compare differences with control (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

BDNF and TrkB expression in the SVZ of the adult mouse

To investigate the involvement of BDNF/TrkB signalling in the SVZ-derived neuroblast migration, we analysed the expression and relative amounts of BDNF and catalytic form of its receptor TrkB by real-time RT-PCR. Tissue microdissections were obtained from two levels: the anterior SVZ, adjacent to the LV (SVZ-LV); and the core of the OB (SVZ-OB; Fig. 1A). The levels of expression of BDNF and TrkB were in the same order of magnitude (ranging from 6 ± 0.5 to 127 ± 22 no. of copies/ng, Fig. 1B and C). Interestingly, for both BDNF and TrkB receptors the transcript expression was lower in the SVZ-LV compared with SVZ-OB extracts. A similar pattern was also observed for BDNF detected by ELISA (Fig. 1D). To exclude that the lower levels of expression found in the SVZ-LV reflect possible 'contamination' of striatal tissue into SVZ-LV samples, we run the same analysis on striatal tissue extracts. The amount of BDNF and TrkB mRNAs was abundantly larger in striatal extracts (BDNF 275 ± 69 no. copies/ng; TrkB 178 ± 99 no. copies/ng) compared with SVZ-LV extracts (BDNF 13 ± 6 no. copies/ng; TrkB 6 ± 0.5 no. copies/ng). Interestingly, the significantly higher amount of BDNF mRNA did not reflect differences in the amount of BDNF protein (11 ± 3 pg/ng striatal tissue vs 8 ± 4 pg/ng SVZ-LV). Together these results support low, if any 'contamination' of striatal tissue into SVZ-LV microdissections.
The distribution of the TrkB receptor protein was analysed by immunofluorescence. According to previous reports on the TrkB expression pattern in the CNS of the adult rat (Yan et al., 1997), widespread distribution of TrkB-immunopositive elements was found in the whole forebrain (not shown). In the SVZ, TrkB immunoreactivity was observed at all levels of the migratory pathway (Fig. 1E–G), with no antero-posterior differences in signal intensity. To identify the phenotype of TrkB-labelled cells in the SVZ, we performed double-immunolabelling reactions using anti-PSA-NCAM antibody to stain SVZ migrating neuroblasts and anti-GFAP to label SVZ astrocytes. As shown in Fig. 1, TrkB-immunopositive cells were double-labelled for PSA-NCAM (Fig. 1H–L), whereas no co-localization was found with GFAP (Fig. 1M–O). These results indicate that the TrkB receptor is expressed by SVZ migrating neuroblasts. Within the OB, besides migrating neuroblasts, TrkB was also localized in numerous cells distributed over the OB layers (i.e. granule cell layer and mitral cell layer), confirming previous results (Yan et al., 1997; Fig. 1G).

BDNF via TrkB receptor enhances migration of neuronal precursors from SVZ explants

To investigate the potential role of BDNF on SVZ neuroblast migration, we adopted an in vitro system, culturing SVZ tissue explants in a matrigel 3D matrix, a well-established method that allows chain migration of neuronal precursors in a way similar to that occurring in vivo (Wichterle et al., 1997). To limit the variability of explants in size and migratory response, samples were obtained from coronal sections taken at the level of the SVZ-RE corresponding to the horizontal arm of the RMS (Fig. 2A). In control conditions, after 24 h, compact chains of migrating neuroblasts were symmetrically distributed around each explant (Fig. 2C). According to our in vivo findings, these cells were double-positive for PSA-NCAM and TrkB (Fig. 2B), demonstrating that SVZ migrating neuroblasts also maintain TrkB receptor expression in vitro.
In order to define whether BDNF influences SVZ neuroblast migration, explants were treated with BDNF (50 ng/mL, Polleux et al., 2002) for 24 h, starting from the plating time. Each explant was fixed at 1 DIV and counterstained with the nuclear dye SYTOX Green (Fig. 2C–E). The area occupied by the SYTOX Green-positive nuclei of the cells migrated out of the explants, here referred to as 'migration area', was quantified. When exogenous BDNF was added to the culture medium, we observed a 2.5-fold increase of the migration area, compared with control explants (Fig. 2C, D and F), suggesting a motogenic effect of this neurotrophin acting

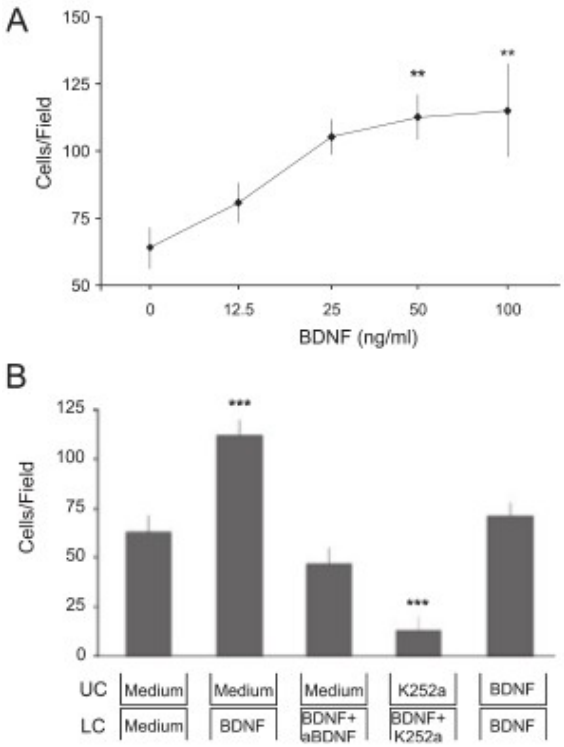

Fig. 3. Brain-derived neurotrophic factor (BDNF) is a chemoattractant for SVZ neuroblasts. (A) Boyden chamber assay on freshly dissociated SVZ cells showing a dose-dependent chemoattractant effect of BDNF. The increase in directional migration was statistically significant from 50 ng/mL of BDNF (~180% of control). (B) The directional migratory effect of BDNF (50 ng/mL) on SVZ-derived cells is neutralized by addition of anti-BDNF blocking function antibody in the lower chamber or by addition of the same concentration of BDNF in both chambers. Addition of TrkB inhibitor K252a (50 nm) significantly reduces migration compared with control conditions. Data (mean ± standard error of the mean) are presented as the number of cells per field. One-way anova followed by Fisher's LSD post hoc was used to compare groups (**P 1⁄4 0.01; ***P < 0.001). LC, lower chamber; UC, upper chamber.

I through activation of TrkB receptors on SVZ precursors. To confirm the involvement of TrkB activation in this process, SVZ explants were treated with Trk receptor tyrosine kinase inhibitor K252a (50 nm; Tapley et al., 1992; Polleux et al., 2002) added to the culture medium either alone or in combination with BDNF. K252a treatment blocks BDNF-induced migration (Fig. 2E and F), leading to values similar to controls, supporting a role for TrkB in this process. Interestingly, incubation of K252a alone induced a reduction of about 40% of the migration area compared with control conditions (Fig. 2F). Migration observed in controls, maintained in serum-free medium, is likely due to the activity of motogenic factors endogenously produced by the tissue explants. According to our results, which show expression of BDNF by SVZ tissue explants (Fig. 1D), this K252a-induced inhibition in basal conditions supports a physiological role for BDNF/TrkB interaction in SVZ neuroblast migration. However, we cannot rule out that this effect also reflects, at least partially, inhibition of other tyrosine kinase receptors sensible to K252a inhibitor (Tapley et al., 1992). To further investigate the motogenic effect of BDNF we evaluated the distribution of cells within the migration area, by quantifying the number of cells that reaches progressive distances (50-μm intervals) from the perimeter of the tissue explants (see Materials and methods for details). BDNF treatment significantly increased the percentages of cells at the different intervals of migration distances calculated within the migratory area (Fig. 2G), but did not affect the maximal distance (250 μm) reached by the migrating neuroblasts at 1 DIV. As for the migration area, also for this analysis, explants treated with BDNF in the presence of K252a gave results similar to controls (Fig. 2G). In order to rule out the possibility that BDNF treatment might indirectly affect the extension of the migration area through activation of cell proliferation or enhancement of cell survival, we evaluated by immunohistochemistry the expression of the endogenous marker of cell

proliferation Ki67 (Scholzen & Gerdes, 2000), and of activated caspase-3, which has been shown to be implicated in programmed cell death (Srinivasan et al., 1998). Both in the absence and presence of exogenous BDNF, Ki67- and caspase-3-positive nuclei were mainly observed in the tissue explants, whereas only rarely were positive nuclei found in cells that had migrated out of the explants (not shown). No differences were observed in the numbers of Ki67- or caspase- 3-positive elements in BDNF-treated explants (8.4 ± 0.2 Ki67 cells/explant, N 1⁄4 9; 46 ± 2 caspase-3 cells/explant, N 1⁄4 9) compared with controls (10 ± 1.7 Ki67cells/explant, N 1⁄4 9; 46 ± 1 caspase-3 cells/explant, N 1⁄4 9). These results indicate that BDNF has no effect on SVZ neuroblasts survival, and confirm previous data showing that BDNF did not affect cell proliferation in SVZ precursors cultured in vitro (Kirschenbaum & Goldman, 1995). Moreover, based on observations on PSA-NCAM-immunostained explants we also managed to exclude a detachment effect, as no differences were observed in the organization of the neuronal chains after BDNF treatment (not shown).

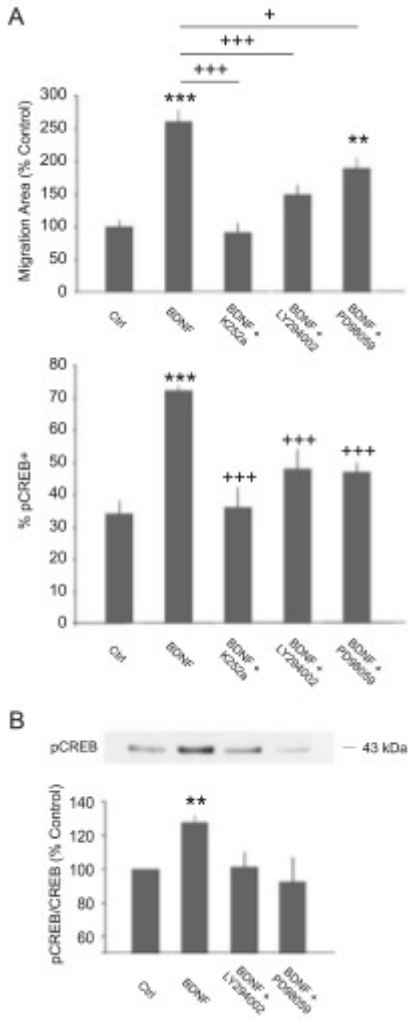

Fig. 4. Brain-derived neurotrophic factor (BDNF) regulates migration of SVZ precursor cells through cAMP response element-binding protein (CREB) activation via PI3-K and MAP-K signalling. (A) At 1 DIV, BDNF-induced migration on SVZ-RE explants is associated with an increase in the percentage of cells immunopositive for pCREB in the migration area. Incubation in the presence of TrkB inhibitor K252a (50 nm), PI3-K inhibitor LY294002 (10 µm) or MAP-K inhibitor PD98059 (10 µm), strongly reduces the BDNF motogenic effect (A) and CREB phosphorylation in migrating cells. (B) pCREB expression evaluated by Western blot analysis on SVZ explants treated for 20 min either with control culture medium, BDNF (50 ng/mL), BDNF in the presence of LY294002 (10 µm), or BDNF in the presence of PD98059 (10 µm). Densitometric analysis shows an increase in CREB phosphorylation in samples treated with BDNF compared with controls. Treatment with PI3-K inhibitor LY294002 and MAP-K inhibitor PD98059 block BDNF-induced phosphorylation of CREB. Data on CREB phosphorylation were normalized against CREB expression

levels. Results are presented as means ± standard error of the mean. One-way anova followed by Fisher's LSD post hoc was used to evaluate significant differences from control (**P 1⁄4 0.01, ***P 1⁄4 0.001) or from BDNF treatment (+ P 1⁄4 0.05, +++ P 1⁄4 0.001).

BDNF is a chemoattractant for SVZ neuroblasts

The experiments described above show that BDNF induces neuroblast migration, contributing to the initiation and maintenance of neuronal movement. As shown by ELISA on SVZ explants, BDNF protein levels are higher in extracts of the SVZ-OB than the SVZ-LV. Thus, in vivo, SVZ neuroblasts migrate along the RMS from the LV, a region of low expression of BDNF, to the OB core, where BDNF reaches higher levels. This pattern of expression is consistent with a role for BDNF in chemoattraction on SVZ neuroblast migration. To investigate this hypothesis, we used the Boyden chamber assay. Dissociated cells from the SVZ-RE were plated in the upper compartments and allowed to migrate through a porous membrane separating the upper and lower compartments. Cells that migrated to the lower side of the membrane were stained with DAPI and counted. Addition of BDNF to the lower compartment of the chamber only, establishes a concentration gradient that mimics the gradient of molecules that cells can encounter in vivo. We observed a dose-dependent increase in the number of cells migrating into the lower compartment following BDNF treatment (Fig. 3A). Statistically significant values were obtained starting from concentrations of 50 ng⁄mL, which induce a twofold increase when compared with medium alone (Fig. 3A and B). Incubation of BDNF in the presence of the blocking function anti- BDNF antibody or K252a inhibitor (50 nm) completely eliminates the BDNF-induced migratory response (Fig. 3B). In accordance with our results obtained on tissue explants, K252a-treated cells had a substantially reduced motility compared with controls (Fig. 3B), supporting the fact that inhibition of TrkB may interfere with the activity of endogenously produced BDNF.
To better investigate whether the effect of BDNF on the motility of the SVZ neuroblasts was specifically directional (chemotaxis), we disrupted the gradient by adding the same concentration of the factor in both chambers. As a result, the number of cells that had migrated to the lower side of the membrane showed values similar to controls (Fig. 3B), confirming the chemoattractive effect of BDNF on these cells.

BDNF stimulates CREB phosphorylation and regulates migration of SVZ neuroblasts via PI3-K and MAP-K signalling pathways

Upon BDNF binding, dimerization and phosphorylation of TrkB receptors lead to activation of multiple downstream pathways which, in turn, activate many substrates including the transcription factor CREB, which is an important regulator of BDNF-induced gene expression (Bonni et al., 1995; Finkbeiner et al., 1997). We have recently demonstrated that CREB is expressed by SVZ-derived neuroblasts, and it is progressively activated through phosphorylation along the migratory pathway (Giachino et al., 2005). To further elucidate the function of BDNF in neuronal migration, and the possible involvement of CREB activation in this process, we analysed the downstream signalling events activated by BDNF in SVZ precursor cells. CREB phosphorylation was evaluated by immunofluorescence on neuronal precursors migrating from SVZ explants at 1 DIV. In control conditions, 34 ± 2% of the cells in the migration area were immunopositive for the phosphorylated form of CREB (pCREB, Fig. 4A). Following BDNF treatment (50 ng⁄mL), a 2.1-fold increase in the percentage of pCREB-immunopositive cells (72 ± 2%) and a parallel 2.5-fold increase in the migration area were observed (Fig. 3A). Both the effects on CREB phosphorylation and cell migration were blocked by incubation with K252a (50 nm, Fig. 3A), thus further supporting a correlation between BDNF-induced CREB activation and migration. To interfere with CREB activation, SVZ explants were incubated in the presence of BDNF (50 ng⁄mL) with inhibitors of two protein kinases involved in CREB phosphorylation (Vlahos et al., 1994; Dudley et al., 1995). To determine whether BDNF-induced CREB phosphorylation is mediated through PI3-K and⁄or

through the MAP-K activator MAP-K kinase (MAP-KK), we used the PI3-K inhibitor LY 294002 (10 μm) or MAP-KK-specific inhibitor PD 98059 (10 μm). Pharmacological blockade of either PI3-K or MAP-KK on SVZ explants significantly reduced CREB phosphorylation (to 48 ± 6% and 47 ± 3%, respectively, Fig. 4A) and impaired neuronal migration (Fig. 4A) compared with BDNF-treated samples. Interestingly, neither MAP-KK inhibitor nor PI3-K inhibitor alone was able to completely eliminate BDNF effects, supporting BDNF activation of CREB through involvement of multiple pathways.

Our results strongly support a correlation between BDNF-induced enhancement of cell migration and CREB activation, suggesting a new role for this transcription factor in neuronal migration. In order to prove that CREB phosphorylation is directly activated by BDNF, we performed acute treatment on freshly dissected SVZ explants. Exposure to BDNF for 20 min led to robust CREB activation, as detected by Western blot using anti-pCREB antibody (Fig. 4B), whereas incubation of BDNF in the presence of PD 98059 or LY 294002 was ineffective in inducing CREB activation (Fig. 4B). These data further support BDNF migratory activity on SVZ neuroblasts being mediated by CREB activation.

Discussion

In this study we demonstrate a role for BDNF in the regulation of SVZ neuroblast migration. BDNF has been previously demonstrated to promote dendritic development of SVZ-derived neuronal precursors (Gascon et al., 2005), as well as being essential for their survival in the OB (Kirschenbaum & Goldman, 1995; Goldman et al., 1997; Linnarsson et al., 2000). Here we show that BDNF expression is not restricted to the OB, but it occurs throughout the whole SVZ system, although both BDNF mRNA and protein are present at higher levels in the SVZ-OB compared with SVZ-LV. We show that similarly to BDNF, its high-affinity receptor TrkB is also expressed along the whole SVZ system, in accordance with previous reports (Zigova et al., 1998). Moreover, by confocal microscopy analysis we demonstrate that TrkB localization throughout the system is specifically found in PSA-NCAM-immunopositive neuroblasts.

This pattern of expression suggests a role for BDNF and TrkB in SVZ neuroblast migration. Consistent with this hypothesis, previous studies reported a substantial increase in the number of newly generated cells within the migratory pathway and in the OB after BDNF intraventricular administration (Zigova et al., 1998) or infection with adenoviral BDNF (Benraiss et al., 2001). Moreover, BDNF via TrkB activation has been previously demonstrated to stimulate migration of cortical interneurons (Polleux et al., 2002) and cerebellar granule cells (Borghesani et al., 2002) during development.

To directly investigate whether BDNF is involved in controlling cell migration in this system, we adopted an in vitro approach using SVZ explants cultured in matrigel. The confocal microscopy analysis on TrkB expression in the explants confirms the in vivo results, showing co-localization for PSA-NCAM and TrkB on migrating neuroblasts, further suggesting TrkB involvement in neuroblast migration.

Tissue explants treated for 24 h in the presence of exogenous BDNF show a dramatic increase in the number of cells migrating out of the explants, and in the percentage of cells that reached higher distances; however, no changes are observed in the maximal distance reached by the migrating cells when BDNF-treated explants are compared with controls. Accordingly, a previous study, screening several molecular factors for modulation of SVZ neuroblast migration, reported no effects of BDNF on the maximal distance that cells had moved away from the perimeter of SVZ explant (Mason et al., 2001).

Besides an inductive role in cell migration, the observed increase in the amount of cells around each explant following BDNF treatment could also partially reflect its effect on cell proliferation or survival. However, this seems unlikely as our data on the expression of the endogenous marker of cell proliferation Ki67 clearly indicate that BDNF treatment has no effect on SVZ progenitor

proliferation, thus confirming previous [^{3} H]thymidine incorporation experiments (Kirschenbaum & Goldman, 1995). Moreover, BDNF has been shown to have an inhibitory effect on proliferation of neural progenitor cells (Cheng et al., 2003; Tervonen et al., 2006). Although a role for BDNF in the survival of SVZ-derived neurons has been previously demonstrated both in vivo (Linnarsson et al., 2000) and in vitro (Kirschenbaum & Goldman, 1995; Goldman et al., 1997), the absence of survival effect on migrating neuroblasts observed in SVZ explants indicates that BDNF can exert different effects on SVZ-derived cells at different maturation stages (immature-migrating cells vs differentiated neurons).

The induced motility on SVZ neuroblasts is eliminated when BDNF is incubated in the presence of K252a, which blocks autophosphorylation of TrkB (Tapley et al., 1992), proving the involvement of these receptors in the migratory response. Interestingly, K252a treatment is also effective in reducing migration in the control condition, in the absence of exogenous BDNF, suggesting that SVZ tissue explants can be an endogenous source for BDNF, according to our results on BDNF protein expression in vivo. Besides acting as classical target-derived trophic factors, neurotrophins have been also suggested to act through paracrine or autocrine mechanisms (Schecterson & Bothwell, 1992; Kokaia et al., 1993; Borghesani et al., 2002; Gascon et al., 2005). Accordingly, our data indicate that BDNF, possibly released by migrating cells, acts to promote migration in an autocrine/paracrine manner. Quantitative analysis on BDNF protein levels in the SVZ-OB and SVZ-LV suggests the occurrence of a gradient along the migratory route, from the LV to the OB. This is consistent with differences in the relative cell composition of the SVZ at the level of the LV (ependymal cells, type B, type C cells and migrating neuroblasts) and OB, where most of the cells are represented by migrating neuroblasts (Alvarez-Buylla & Garcia-Verdugo, 2002).

To test whether a gradient of BDNF is able to induce directional movement of SVZ precursors, we used a Boyden chamber assay. We show that BDNF acts as a chemoattractant, significantly increasing neuroblast directional migration. Incubation with anti-BDNF blocking function antibody or K252a inhibitor eliminates BDNF-induced chemotaxis. In addition, K252a treatment alone substantially reduces migration compared with controls, similar to results obtained on SVZ explants, further supporting the likelihood of inhibition of TrkB function interfering with the biological activity of endogenously produced BDNF. These overall results demonstrate that BDNF, besides the motogenic effect, can have a chemoattractive function on SVZ neuroblasts through an autocrine/paracrine mechanism.

Target-derived chemoattractive cues play a critical role in directing SVZ neuroblasts toward the OB (Liu & Rao, 2003). However, after removal of the OB, SVZ cells continue to migrate anteriorly (Kirschenbaum et al., 1999), indicating that OB is not essential for the directional migration of SVZ neuroblasts. Thus, local signals within the RMS may contribute to providing a directional cue for migrating precursors, and BDNF can represent one of such signals.

Our investigations into the downstream signalling events promoted by BDNF suggest that activation of the transcription factor CREB controls BDNF-induced neural migration. CREB is a major mediator

of neuronal neurotrophin responses (Finkbeiner et al., 1997; Finkbeiner, 2000), and it has been demonstrated to control several steps of the neurogenic process, including proliferation, differentiation and survival in the adult hippocampus and SVZ (Nakagawa et al., 2002a,b; Kodama et al., 2004; Giachino et al., 2005). Although a role for this transcription factor in cell migration has not been demon

strated yet, there is increasing evidence supporting its involvement in this process. Indeed, we have recently shown that CREB is expressed by SVZ-derived neuroblasts, and it is progressively activated through phosphorylation along the migratory pathway (Giachino et al., 2005). Furthermore, following peripheral afferent denervation of the OB, downregulation of CREB activation in the SVZ-OB parallels impairment in radial cell migration (Giachino et al., 2005). Here we show that acute (20 min) BDNF treatment induces robust CREB phosphorylation in SVZ explants. At 1 DIV following BDNF exposure, parallel to the increase of the migration area, we

found a significant increase in the percentage of pCREB-immunopositive migrating neuroblasts. Moreover, our data indicate that CREB phosphorylation requires TrkB activation as treatment with K252a completely blocks CREB activation.
Following BDNF stimulation of TrkB receptors, multiple signalling pathways are activated (Huang & Reichardt, 2003) and may converge on CREB phosphorylation (Finkbeiner et al., 1997; Finkbeiner, 2000). We found that in SVZ explants, PI3-K and MAP-K are both required for BDNF-induced CREB activation and cell migration. Previous studies demonstrated that PI3-K but not MAP-K signalling is required in the control of cortical interneuron migration by neurotrophins (Polleux et al., 2002), suggesting that different pathways may be implicated in the same biological process in different systems. Activation of MAP-K together with PI3-K has also been demonstrated necessary for reelin-dependent detachment of SVZ neuroblast chains in the OB (Simo et al., 2006). Interestingly, detachment occurs after specific activation of the reelin transduction cascade, whereas BDNF, which independently activates MAP-K in the same cells, is unable to cause detachment (Simo et al., 2006), in agreement with our observations. Thus independent activation of the same signalling pathways in the SVZ neuroblasts can induce diverse biological responses.
These overall results indicate that multiple molecular cues and signalling cascades are specifically required to control different steps of adult SVZ neurogenesis. Within this complex scenario our data support a functional implication of BDNF through activation of CREB via PI3-K and MAP-K in the regulation of neuroblast migration.

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Abbreviations

BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CREB, cAMP response element-binding protein; DIV, days in vitro; DMSO, dimethylsulphoxide; ECL, enhanced chemiluminescence; ELISA, enzyme- linked immunosorbent assay; GFAP, glial fibrillary acidic protein; LV, lateral ventricle; MAP-K, mitogen-activated protein kinase; MAP-KK, MAP-K kinase; OB, olfactory bulb; P, postnatal day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; PI3-K, phosphati- dylinositol 3-kinase; PSA-NCAM, polysialic acid neural cell adhesion molecule; RE, rostral extension; RMS, rostral migratory stream; RT, reverse transcriptase; SDS, sodium dodecyl sulphate; SmRNA, standard mRNA; SVZ, subventricular zone; TBST, Tris-buffered saline Tween-20.

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