Development 129, 000-000 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV4601

BDNF stimulates migration of cerebellar granule cells

Paul R. Borghesani[1,3,*], Jean Michel Peyrin[2,*], Robyn Klein[4,*], Joshua Rubin[1], Alexandre R. Carter[1,3], Phillip M. Schwartz[1], Andrew Luster[4], Gabriel Corfas[2,†] and Rosalind A. Segal[1,3,5,‡]

[1]Department of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA
[2]Division of Neuroscience, The Children's Hospital, Boston, MA 02115, USA
[3]Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
[4]Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Charlestown, MA 02129, USA
*The first three authors contributed equally
†The last two authors contributed equally
‡Author for correspondence (e-mail: rosalind_segal@dfci.harvard.edu)

Accepted 17 December 2001

SUMMARY

During development of the nervous system, neural progenitors arise in proliferative zones, then exit the cell cycle and migrate away from these zones. Here we show that migration of cerebellar granule cells out of their proliferative zone, the external granule cell layer (EGL), is impaired in *Bdnf*$^{-/-}$ mice. The reason for impaired migration is that BDNF directly and acutely stimulates granule cell migration. Purified *Bdnf*$^{-/-}$ granule cells show defects in initiation of migration along glial fibers and in Boyden chamber assays. This phenotype can be rescued by exogenous BDNF. Using time-lapse video microscopy we find that BDNF is acutely motogenic as it stimulates migration of individual granule cells immediately after addition. The stimulation of migration reflects both a chemokinetic and chemotactic effect of BDNF. Collectively, these data demonstrate that BDNF is directly motogenic for granule cells and provides a directional cue promoting migration from the EGL to the internal granule cell layer (IGL).

Movies available on-line

Key words: BDNF, Granule cells, Cell migration, CNS, Mouse

INTRODUCTION

Development of the central nervous system (CNS) follows a common pattern of localized proliferation followed by cell migration and differentiation. In the cerebral cortex, progenitors proliferate in the ventricular zone and then migrate into the cortical layers where they differentiate into a vast array of cortical cell types. In the cerebellar cortex this general pattern is modified (Altman and Bayer, 1997). Cerebellar granule cell progenitors are produced in the rhombic lip, then migrate over the cerebellar primordium to form a secondary proliferative zone, the external granule cell layer (EGL). During early post-natal development, committed granule cell precursors in the outer zone of the EGL proliferate, then exit the cell cycle, differentiate and migrate through the molecular layer (ML) past the Purkinje cells to their destination, the internal granule cell layer (IGL).

The processes of granule cell differentiation and migration depend on many extracellular factors (Hatten, 1999). Among these are the neurotrophins. Brain-derived neurotrophic factor (BDNF) is expressed in cerebellar granule cells (Rocamora et al., 1993; Wetmore et al., 1990) and the level of BDNF expression increases during development. Granule cells also express the high affinity BDNF receptor, TrkB (NTRK2; Klein et al., 1990; Segal et al., 1995). Activated Trk receptors can be detected in the EGL of wild-type mice, and receptor activation is reduced in *Bdnf*$^{-/-}$ mice, indicating that TrkB receptors function during early granule cell development (Schwartz et al., 1997). Thus BDNF is one of the factors that could regulate early granule cell development.

It is well established that BDNF promotes granule cell survival (Lindholm et al., 1993; Schwartz et al., 1997; Segal et al., 1992) and can stimulate axonal outgrowth by these cells (Gao et al., 1995; Segal et al., 1995). In accordance with these well established functions of BDNF, analysis of *Bdnf*$^{-/-}$ mice revealed impaired survival of granule cells (Schwartz et al., 1997). However, analysis of the mutant mice also resulted in a surprising finding – although there is increased granule cell apoptosis in the absence of BDNF, the EGL is actually thicker in mutant than in wild-type littermates (Jones et al., 1994; Schwartz et al., 1997). There are several potential explanations for this finding. The layering defect could result from delayed differentiation of granule cells. This explanation accords well with the known ability of neurotrophins to promote neuronal differentiation (Henderson, 1996). Alternatively, this defect in layer formation could reflect a novel role for BDNF in migration of granule cells.

Here we show that BDNF stimulates migration of granule cells. In the absence of BDNF, migration of granule cells is impaired both in vivo and in vitro. The impaired migration of granule cells can be rescued by exogenous BDNF, indicating that it is a direct result of the lack of BDNF. Acute addition of BDNF

immediately stimulates granule cells to begin migration, indicating that BDNF is a motogenic factor. Furthermore, BDNF produces a directional cue that prompts the radial migration of granule cells. The accumulating evidence that BDNF exerts motogenic and chemotactic effects and thereby regulates migration adds to a growing and diverse list of neurotrophin functions.

MATERIALS AND METHODS

Reagents

The *Bdnf*$^{-/-}$ mice (Ernfors et al., 1994) used in these experiments were obtained from Jackson Laboratory. PCR-based genotyping was done as described previously (Schwartz et al., 1997). Recombinant hBDNF used in these experiments was obtained from Dr A. Welcher, Amgen or from Peprotech. The NEUROD1 probe for in situ hybridization corresponds to 436-844 (Miyata et al., 1999; Lee et al., 2000) and was obtained from Dr Qiu Fu Ma (Dana-Farber Cancer Institute). The probe for ATOH1 (MATH1) (Kenney and Rowitch, 2000) was obtained from Dr David Rowitch (Dana-Farber Cancer Institute). Antibodies used were a polyclonal antibody to ZIC generated using the carboxy-teminal peptide (AVHHTAGHSALSSNFEWYV), antibodies to BDNF (Santa Cruz, and Promega), monoclonal antibody to bromodeoxyuridine (Boehringer Mannheim), anti-SNAP25 (Chemicon).

In vivo BrdU labeling and immunohistochemistry

Mice were injected subcutaneously with 50 mg/kg of 5-bromo-2′-deoxyuridine (Sigma) and the animals were sacrificed at the indicated times. The pups were perfused with 4% paraformaldehyde and 14 μm sagittal cryostat sections were prepared. These were permeabilized in 0.4% Triton X-100, treated for 1 hour at 37°C with 2 M HCl followed by two 10-minute washes in 0.1 M sodium borate and stained with anti-BrdU antibody (Boehringer Mannheim) at 1:50, and visualized with Cy3- or HRP-linked secondary antibodies (Jackson Laboratories). In migration experiments the number of labeled cells/mm in each cortical layer was counted in five non-adjacent mid-sagittal sections on folia 6 within the primary fissure and on folia 9 in the secondary fissure. Four sets of *Bdnf* littermates (counted at 42 and 96 hours post-labeling) were used.

In vitro migration assays

Radial migration assays were carried out using primary glial and neuronal cells purified from P5-P7 wild-type and *Bdnf*$^{-/-}$ cerebella as described by Hatten (Hatten, 1985) with a few modifications (Rio et al., 1997). Cerebella were collected and the meninges were removed. Tissue blocks were incubated in 1% trypsin (Sigma) with 0.1% DNaseI (DNase, Worthington) in PBS for 10 minutes at 37°C and triturated in Ca^{2+}/Mg^{2+}-free HBSS (Gibco BRL) with 0.1% DNase using fire-polished Pasteur glass pipettes. The cell suspension was layered on top of a two step Percoll gradient (35/60%, Pharmacia Biotech) in PBS. After a 3,000 rpm centrifugation at 4°C for 10 minutes, the glia rise to the top of the gradient, and the neurons are found in the 35-60% interface. Cell fractions were washed once in PBS and twice in basal medium Eagle (BME) before use. Previous studies have demonstrated that the granule cells prepared and plated in this way are derived from the EGL, and predominantly express the early transcription factor ATOH1 (MATH1) (Hatten, 1985; Kenney and Rowitch, 2000).

For glial cultures, cells were resuspended in BME with 10% fetal bovine serum (FBS), 0.1% glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were preplated for 25 minutes on an uncoated tissue culture flask to remove contaminating fibroblasts, and transferred to a tissue culture flask coated with 25 μg/ml of poly-D-lysine (Collaborative) for 1 hour to allow glial attachment; the flask was rinsed and left in the same medium. Glial cells were used for no more than two passages.

For granule cell cultures the cells were resuspended in the same media as above. Glia were removed by plating the cells on tissue culture dishes coated with 25 μg/ml poly-D-lysine 3 times for approximately 1 hour each. After each preplating period, the non-adherent cells were gently removed. The purified neurons were plated on top of the glial cells at a ratio of 5-10 neurons per glial cell and cultures were maintained for 20-24 hours prior to time-lapse analysis.

Time-lapse video microscopy and quantification of migration

Cultures, supplemented with 10 mM Hepes and covered with a layer of mineral oil to prevent evaporation, were placed in a Leiden microincubator (Medical Systems Corp.) and kept at 35°C (Narishige MS-C temperature controller). Granule cells, identified by their characteristic cell body and nucleus size and shape, were chosen for imaging if they were attached to a glial cell with a radial process greater than 50 μm. In each experiment, 20 different fields containing at least one neuron attached to a radial glial fiber were recorded. The following variables were measured: (1) the total number of neurons; (2) the percentage of neurons that attached to radial glia; (3) the migratory index, the percentage of neurons attached to radial glia that migrated at more than 10 μm/hour; (4) the average speed of migration of the migrating neurons. Speed of migration was calculated by measuring the position of individual granule cells along glial fibers every 4 minutes over 120-180 minutes using Openlab 2.06 imaging software. Data shown represent the average speed of migrating cells over the course of 120-180 minutes of observation.

Chemotaxis assay

Purified populations of granule cells were obtained from post-natal day 8 (P8) *Bdnf*$^{+/+}$ and *Bdnf*$^{-/-}$ littermates. Purified cells were resuspended at 2-5×10^6 cells/ml in serum-free DMEM (supplemented with N2 growth medium (Gibco, Grand Island, NY) and 20 mM KCl), for use in chemotaxis assays. The in vitro migration of granule cells was assessed using laminin (Sigma; 20 μg/ml) coated polyvinylcarbonate-free membranes (Neuroprobe Inc., Gaithersburg, MD) with 12 μm pore size in modified Boyden chambers as previously described (Garcia-Zepeda et al., 1996; Klein et al., 2001). Briefly, 50 μl of a solution containing 7×10^6 cells/ml in serum-free DMEM was placed in the upper chamber. BDNF (30 ng/ml) in serum-free DMEM was added to lower chambers alone, or to both chambers. After overnight incubation at 37°C in 8% CO_2, the upper surface of membranes were scraped free of cells and debris, membranes were air-dried, then fixed and stained using Dif-quik cell fixation and staining kit (Dade Behring Inc., Newark, DE). Cells that had migrated through pores and adhered to the membrane were analyzed under high-power light microscopy and counted in five adjacent high-power fields (area of HPF=0.78 mm^2; area of each filter=7.07 mm^2). Experiments were performed in duplicate or triplicate and data are expressed as numbers of cells per high-power field (cells/HPF)±s.e.m. Using cells from wild-type animals in control medium 535 cells per HPF migrated into the lower chamber, corresponding to 1.4% of plated cells. In these short term assays BDNF does not affect proliferation or cell number (P. R. B. and R. A. S., unpublished observations). Data were analyzed for statistical significance between groups using Student's *t*-test.

RESULTS

Granule cell migration is impaired in *Bdnf*$^{-/-}$ mice

In the developing cerebellar cortex of *Bdnf*$^{-/-}$ mice the EGL is thicker than in wild-type littermates (Jones et al., 1994; Schwartz et al., 1997). We previously demonstrated that at P8, the time of peak proliferation in the EGL (Fujita et al., 1966), there is no difference in EGL thickness (*Bdnf*$^{+/+}$, 35±5 μm; *Bdnf*$^{-/-}$, 35±3 μm), while at P14 the EGL is significantly thicker

in the mutant (*Bdnf*$^{+/+}$, 9±1 μm; *Bdnf*$^{-/-}$, 14±1 μm) (Schwartz et al., 1997). Analysis of an intermediate developmental stage, P11, reveals that the EGL in wild-type mice is declining rapidly in size, while the EGL in mutant mice shows a slower decrease in thickness (*Bdnf*$^{+/+}$, 19±2 μm; *Bdnf*$^{-/-}$: 26±3μm). To determine if altered migration contributes to this developmental abnormality of cerebellar cortical layering, we labeled a cohort of granule cells born on P11 with a pulse of BrdU and followed migration of these cells over the next 4 days (Fig. 1A). At 18 (data not shown) and 26 hours post-labeling, BrdU-labeled granule cells had not yet begun to migrate and remained in the EGL of both wild-type and *Bdnf*$^{-/-}$ mice. The first migrating cells were seen in the molecular layer 34 hours post-labeling in wild-type mice but not until 42 hours post-labeling in *Bdnf*$^{-/-}$ mice (Fig. 1B). By 96 hours post-labeling, 97% of the BrdU+ granule cells had migrated into the IGL in wild-type mice. In contrast, in *Bdnf*$^{-/-}$ mice only 45% of the labeled cells had successfully migrated into the IGL while 40% of the BrdU+ granule cells remained in the EGL (Fig. 1C). These data demonstrate that granule cell exodus from the EGL is aberrant in *Bdnf*$^{-/-}$ mice and indicate that BDNF plays a role in regulating granule cell movement in vivo.

Granule cells in the EGL of *Bdnf*$^{-/-}$ mice are able to differentiate

It is possible that the impaired migration observed in the *Bdnf*$^{-/-}$ cerebellum is secondary to a failure in the granule cell differentiation program, which includes migration. To determine whether the migration defects of *Bdnf*$^{-/-}$ granule cells reflect a block or delay of differentiation, we examined expression of a number of granule cell differentiation markers (Fig. 2). During development, granule cells progress from precursors that express the HLH transcription factor ATOH1 (Helms et al., 2000), to fully differentiated neurons that express the basic helix-loop-helix transcription factor NEUROD1 (Lee et al., 2000; Miyata et al., 1999) and the zinc finger transcription factors ZIC1, 2, and 3 (Aruga et al., 1994; Aruga et al., 1996). The more differentiated cells also express synaptic proteins including SNAP25 (Goutan et al., 1999). As shown in figure 2, there are no differences in the cerebellar expression of these markers between wild-type and *Bdnf*$^{-/-}$ mice. Thus, granule cell migration is impaired in *Bdnf*$^{-/-}$ mice, and this does not reflect a general defect of differentiation.

BDNF stimulates initiation of migration

Abnormal movement of granule cells in vivo could reflect deficits in a number of different cellular processes, including changes in initiation, progression or cessation of migration. To determine how granule cell migration is disrupted by BDNF deficiency, we turned to an in vitro assay that recapitulates the radial mode of migration along glial cells. In this assay system, freshly dissociated, purified granule cells derived from the EGL (Hatten, 1985) are plated on top of established glial cell cultures. In the presence of granule cells, glia assume a radial morphology and serve as substrata for the migrating neurons whose movement along these glial fibers can then be quantified (Rio et al., 1997). Here, granule cells purified from wild-type and *Bdnf*$^{-/-}$ littermates were plated on top of syngenic glial cultures. Twenty-four hours later migration was analyzed by time-lapse microscopy during a 2-hour time period (Fig. 3). Data shown represent the combined analysis of over 1000 cells of each genotype analyzed over the course of three independent experiments.

Using time-lapse video microscopy, we measured several distinct aspects of migration. We determined the percentage of neurons that attach to glial processes, the migratory index (defined as the percentage of neurons attached to radial glia that move at least 10 μm in the course of 1 hour of observation), and the speed of migration (see Materials and Methods). We found that wild-type and *Bdnf*$^{-/-}$ granule cells survived equally well and that granule cells of both genotypes induced a radial morphology of the glial cells. Moreover, equal proportions of wild-type and *Bdnf*$^{-/-}$ granule cells attached to the radial glia (Fig. 3A). Strikingly, the migratory index was dramatically reduced in the *Bdnf*$^{-/-}$ cultures: while 42% of wild-type granule cells that were attached to radial glia actively migrated, only 18% of *Bdnf*$^{-/-}$ granule cells attached to radial glia did so (Fig. 3B). However, when the *Bdnf*$^{-/-}$ cells migrated, they did so at the same average speed as wild-type cells (Fig. 3C). Thus individual *Bdnf*$^{-/-}$ granule cells show a decreased migratory index, indicating that there is deficient initiation of radial migration in vitro as well as in vivo.

To confirm that the difference in migration is due to a lack of BDNF, we tested the ability of exogenous BDNF to rescue this defect. BDNF (5 ng/ml) was added to the granule cell/glial cell co-cultures at the time of plating and migration was recorded 24 hours later. Exogenous BDNF increased the migratory index in both wild-type and mutant cultures, resulting in the complete elimination of any difference in migration between the two genotypes (Fig. 4). BDNF did not affect the ability of granule cells of either genotype to attach to glial fibers, nor did it affect the average speed of migration. Furthermore, in this short time period, BDNF did not alter the ability of granule cells to survive. Thus BDNF specifically rescued the defect in initiation of migration that was seen in mutant cells.

In these experiments, exogenous BDNF was added throughout the co-culture period. To determine whether acute exposure to BDNF can modulate granule cell migration, *Bdnf*$^{-/-}$ granule cells were plated on glial cells, and 24 hours later migration was analyzed by time-lapse microscopy for 90 minutes. BDNF was then added to a concentration of 10 ng/ml, and migration of the same cells was analyzed for a second 90 minute period. We found that stationary granule cells began to move 10-20 minutes after addition of BDNF (see Fig. 5A and the Quicktime movie: http://dev.biologists.org/supplemental/). Examination of more than 200 cells over two independent experiments showed that BDNF, but not vehicle control, increased the migratory index of granule cells in this short time period. The acute ability of BDNF to increase migration of granule cells indicates that BDNF is motogenic for granule cells. In summary, we have shown that initiation of granule cell migration is impaired in the absence of BDNF, and exogenous BDNF rapidly induces stationary cells to begin migration.

BDNF directly stimulates granule cell migration

The experiments described above could not determine whether BDNF acts directly on the granule cells to induce movement, or acts on the radial glial fibers to make them better substrates for migration. Furthermore, since these cultures contain both glia and granule cells, we could not determine whether BDNF produced by granule cells themselves stimulates migration. To determine whether BDNF produced by granule cells acts directly

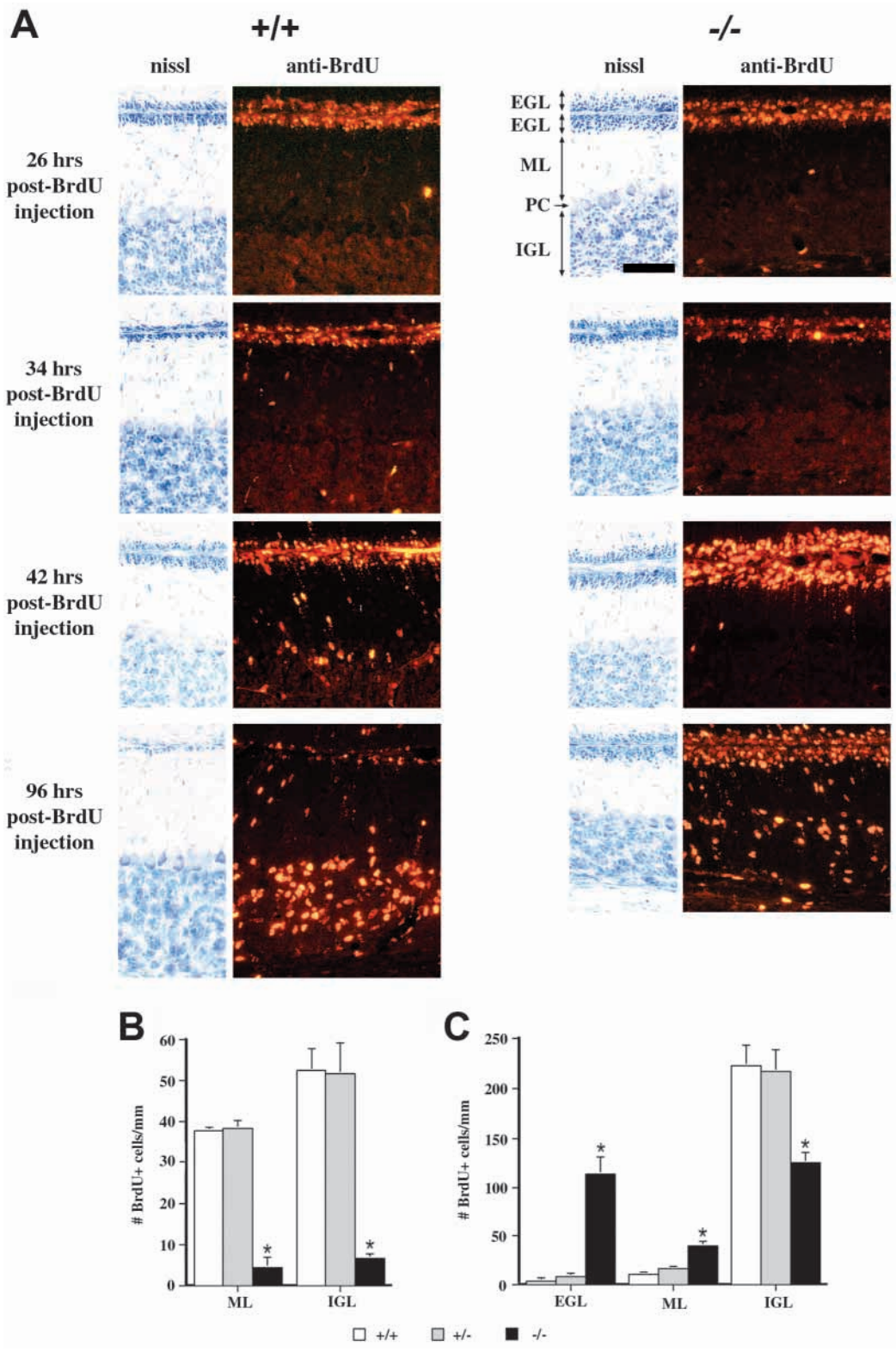

Fig. 1. Granule cell migration out of the EGL is delayed in *Bdnf*$^{-/-}$ mice. **(A)** Dividing granule cells in P11 wild-type and *Bdnf*$^{-/-}$ littermates were labeled by systemic injection of 50 mg/kg BrdU. Cerebellar tissue was then collected and processed for BrdU immunohistochemistry at the indicated times post-injection. Adjacent sections were stained with Cresyl Violet. EGL, external germinal layer; ML, molecular layer; PC, Purkinje cell layer; IGL, internal granule cell layer. Scale bar, 50 μm. (B) At 42 hours post-BrdU labeling, there is a significant reduction in the number of granule cells in the molecular layer and IGL in *Bdnf*$^{-/-}$ mice. **P*<0.005 by two-tailed *t*-test. (C) At 96 hours post-BrdU labeling, fewer granule cells have migrated into the IGL while more BrdU-positive cells remain in the EGL and molecular layer of *Bdnf*$^{-/-}$ mice. The total number of labeled granule cells did not differ between genotypes. **P*<0.005 by two-tailed *t*-test.

on granule cells to promote migration in an autocrine/paracrine manner, we measured the movement of purified granule cells isolated from mutant and wild-type cerebella in Boyden chamber assays (Garcia-Zepeda et al., 1996; Klein et al., 2001). In this assay, purified granule cells are plated on one side of a porous membrane and migration through the membrane into a second compartment is quantified. In the absence of exogenous BDNF, purified *Bdnf*$^{-/-}$ granule cells had a substantially reduced basal motility as compared to wild-type cells (Fig. 6A). This defect in migratory behavior was rescued when BDNF was added to both compartments of the Boyden chamber. Thus, the Boyden chamber experiments confirm that BDNF produced by the granule cells contributes to normal levels of migration. Importantly, these experiments also demonstrate that the defect in migration can be rescued by exogenous BDNF that acts directly on granule cells.

The levels of *Bdnf* mRNA in the developing cerebellum are higher in the IGL than in the EGL (Rocamora et al., 1993), since expression increases as granule cells mature (Maisonpierre et al., 1990). We carried out immunohistochemical studies to examine the distribution of BDNF protein in the developing cerebellum at P7 when granule cells are actively migrating. The antibody to BDNF used is highly specific, and does not stain sections of *Bdnf*$^{-/-}$ mice (Fig. 8) (Schwartz et al., 1997). A similar pattern of staining was seen with a second antibody to BDNF (data not shown). As shown,

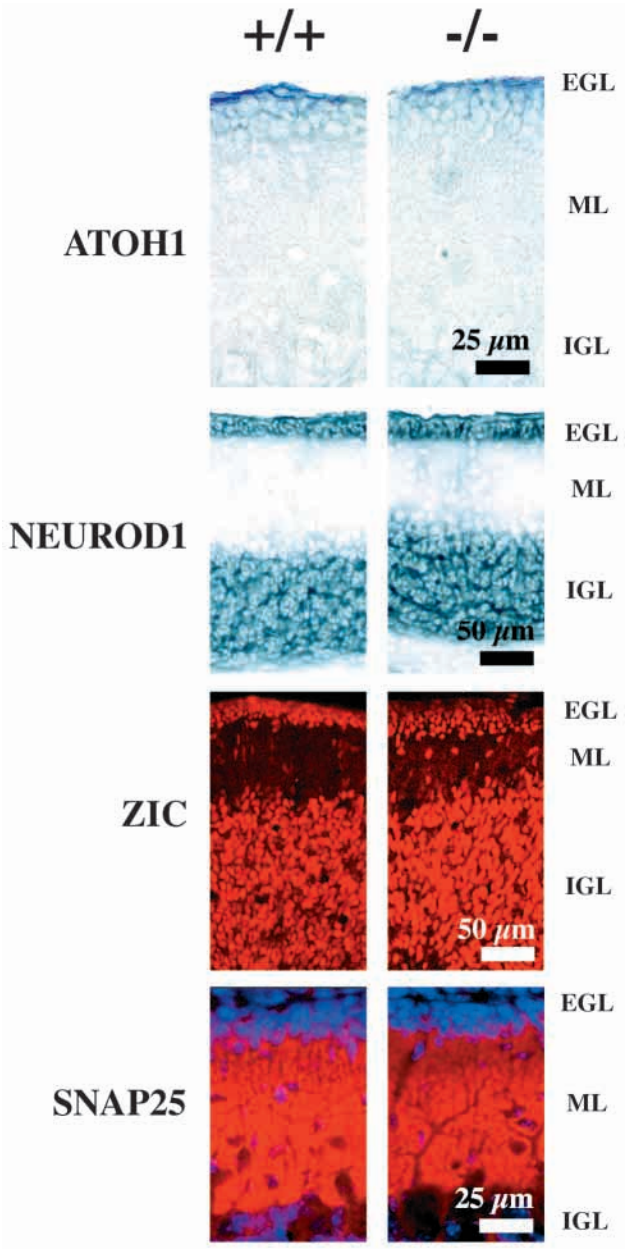

Fig. 2. Granule cells differentiate appropriately in *Bdnf*$^{-/-}$ mice. Mid-sagittal cerebellar sections from P12 wild-type and *Bdnf*$^{-/-}$ mice were processed for in situ hybridization using an antisense probe for the early granule cell marker, ATOH1(Math1), or the marker of later differentiation, NEUROD1. Parallel sections were processed for immunohistochemistry using antibodies to ZIC or SNAP25 (double labeled with DAPI, blue). Granule cells in the EGL and IGL of *Bdnf*$^{-/-}$ mice show appropriate patterns of expression of all markers.

there is a graded expression of BDNF protein that reflects the mRNA expression pattern: BDNF protein is greater in the IGL and molecular layer than in the EGL. The more intense immunostaining seen in the molecular layer compared to the IGL may reflect intracellular trafficking of BDNF protein from granule cell bodies to the parallel fiber axons, and the subsequent uptake of BDNF protein by Purkinje cell dendrites. Thus, in vivo, granule cells migrate from the EGL, a region of low BDNF expression, towards a higher level of BDNF protein.

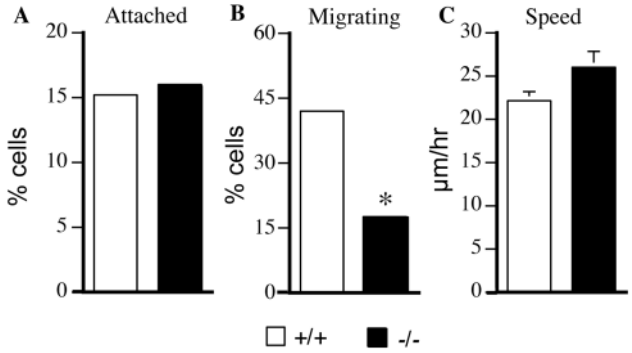

Fig. 3. Purified *Bdnf*$^{-/-}$ granule cells have a decreased migratory index. Freshly isolated wild-type and *Bdnf*$^{-/-}$ granule cells were plated on previously established wild-type and *Bdnf*$^{-/-}$ glial cultures, respectively. After 1 day in vitro, attachment and migration were analyzed by time-lapse video microscopy. One frame every 3 minutes was recorded for 90 minutes. (A) The same proportion of wild-type (230 of 1508) and *Bdnf*$^{-/-}$ (205 of 1280) granule cells attached to glial processes. (B) The proportion of attached *Bdnf*$^{-/-}$ (36 of 205) granule cells that migrated along glial fibers (the migratory index) was significantly lower than that for wild-type (96 of 230) granule cells. *P<0.0001 by χ^2 analysis. (C) Once moving, the migratory rate was the same in wild-type and *Bdnf*$^{-/-}$ cells. White bars, wild-type animals; black bars, *Bdnf*$^{-/-}$ littermates.

In all the experiments thus far, granule cells were stimulated by exogenous BDNF applied uniformly. To determine whether a gradient of BDNF, as occurs in vivo, stimulates directed movement of granule cells, we analyzed the chemotactic effects of BDNF on wild-type granule cells using the Boyden chamber assay. Addition of BDNF to the lower compartment only, establishing a BDNF concentration gradient, increased migration approximately two-fold when compared to medium alone (Fig. 6B). Furthermore, larger numbers of granule cells migrated in response to a gradient of BDNF than in response to a uniform distribution of BDNF, consistent with prior studies (Lu et al., 2001). This indicates that BDNF can function as a chemotactic factor for wild-type granule cells that are competent to migrate.

We conclude that BDNF has a dual role in stimulating granule cell migration. Endogenous BDNF, acting in a paracrine or autocrine fashion, promotes the initiation of migration. Purified granule cells from animals that lack BDNF exhibit defective migration, and this defect can be rescued by acute application of exogenous factor. In addition, a gradient of BDNF stimulates directed movement of granule cells. Thus BDNF is both a motogenic and a chemotactic factor for granule cells.

DISCUSSION

Neurotrophins regulate survival and differentiation of neurons in the peripheral and central nervous systems. We show that BDNF has an additional function in stimulating migration. In vitro, *Bdnf*$^{-/-}$ granule cells initiate migration poorly and exogenous BDNF induces both wild-type and *Bdnf*$^{-/-}$ cells to migrate. In vivo, a lack of BDNF results in impaired migration of granule cells. These data indicate that BDNF has a critical role in stimulating granule cell migration during cerebellar development.

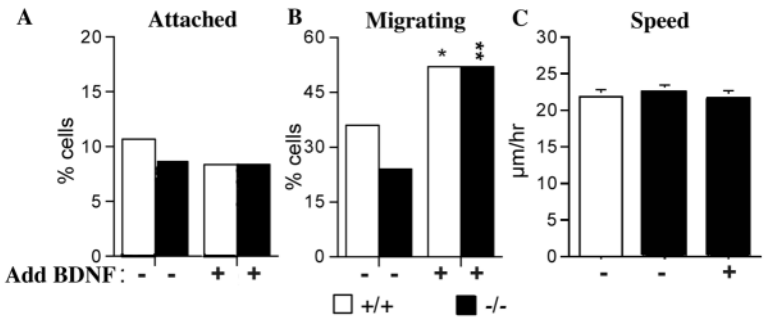

Fig. 4. BDNF induces granule cells to initiate migration. (A) The same proportion of wild-type (124 of 1163 control; 144 of 1720 with added BDNF) and *Bdnf*$^{-/-}$ (103 of 1195 control; 69 of 823 with added BDNF) attached in the absence or presence of exogenous BDNF. (B) Exogenous BDNF increases the migratory index in cultures of both wild-type (45 of 124 control; 75 of 144 with added BDNF) and *Bdnf*$^{-/-}$ (25 of 103 control; 36 of 69 with added BDNF) cells, restoring migration of mutant cells to wild-type levels. $^{**}P<0.01$ and $^{***}P<0.0002$ by χ^2 analysis. (C) BDNF does not affect the speed of granule cell migration. White bars, wild-type animals; black bars, *Bdnf*$^{-/-}$ littermates.

During prenatal development, granule cell precursors migrate from the rhombic lip to the EGL. Then, in a second wave of migration, granule cells attach to radial glia and migrate inwards from the EGL to the IGL. During this radial migration, the granule cells traverse three distinct cerebellar laminae. First they begin to migrate and leave the EGL, second they cross the molecular layer and finally they arrive in the IGL. Using BrdU labeling to visualize a cohort of granule cells, we find that there is a significant delay in the migration of granule cells out of the EGL in *Bdnf*$^{-/-}$ mice (Fig. 1). This in vivo defect in the initiation of radial migration can be recapitulated in vitro, both in a radial migration assay and in a Boyden chamber migration assay. Exogenous BDNF rescues the migration defect of mutant cells. Granule cells of the mutant mice have been subject to chronic BDNF deprivation, and so could exhibit an aberrant response to BDNF. Therefore, an important feature of the data (Fig. 4) is that exogenous BDNF also stimulates radial migration of wild-type cells, by increasing the migratory index beyond the basal level. This gain of function experiment complements the results obtained with the mutant cells, and indicates a specific role for BDNF in promoting the initiation of migration. The rapidity with which BDNF stimulates migration provides further evidence that this is a direct effect. This motogenic or chemokinetic effect was observed in cells exposed to a uniform distribution of BDNF. Taken together, these data indicate a specific role for BDNF in inducing granule cells to initiate radial migration.

In vivo, BDNF mRNA and protein is expressed at higher levels in the IGL than in the EGL (Fig. 7) (Rocamora et al., 1993). Thus the developing granule cells migrate up a BDNF concentration gradient. This gradient may provide one of the directional cues responsible for the inward migration of granule cells along radial glia. Using a Boyden chamber assay, we have shown that a gradient of BDNF can directly induce granule cell chemotaxis. In agreement with our results, others have also found that BDNF acts as a chemoattractant for purified wild-type granule cells (Lu et al., 2001). Thus, BDNF provides granule cells with both an impetus to initiate migration and a directional cue.

Surprisingly, previous studies found that BDNF did not stimulate granule cell migration away from cerebellar explants or reaggregated cells (Tanaka et al., 2000), although K252a, a selective inhibitor of neurotrophin receptors (Tapley et al., 1992), reduced migration (Kobayashi et al., 1995). There are several explanations for the differences in results. Using Boyden chamber assays, we found that a uniform distribution of exogenous BDNF only stimulates chemokinesis of mutant cells that produce no endogenous factor, and does not stimulate chemokinesis of wild-type granule cells. This suggests that when cerebellar cells are cultured at high density, the endogenous BDNF produced by granule cells is sufficient for movement. In the studies cited

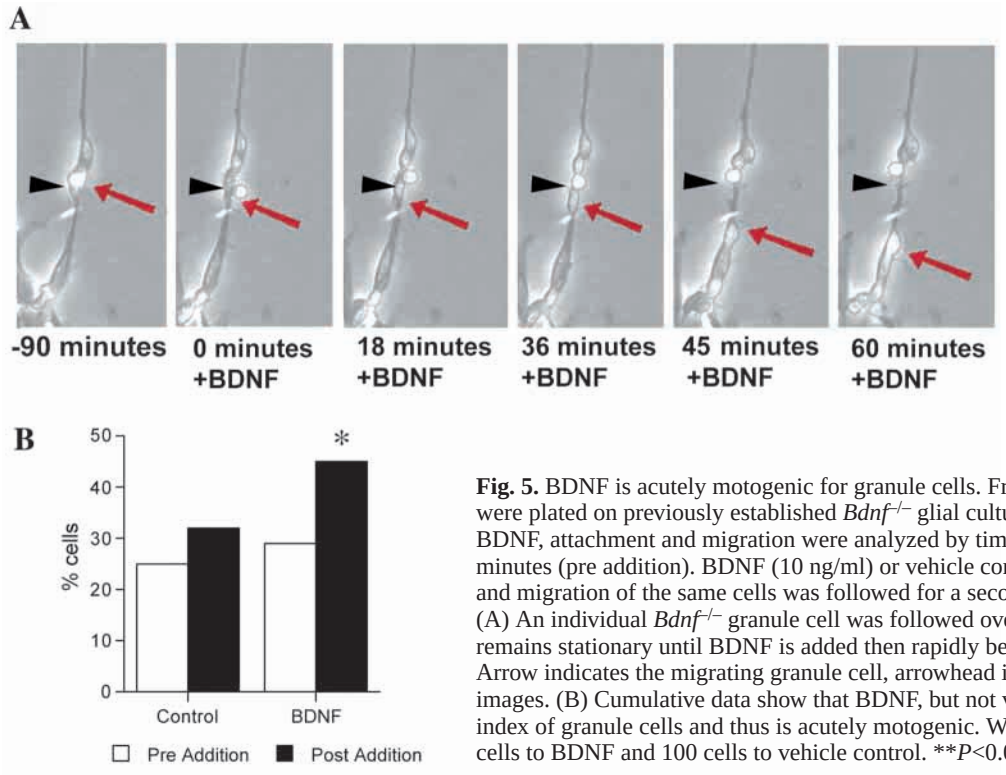

Fig. 5. BDNF is acutely motogenic for granule cells. Freshly isolated *Bdnf*$^{-/-}$ granule cells were plated on previously established *Bdnf*$^{-/-}$ glial cultures. After 1 day in vitro without added BDNF, attachment and migration were analyzed by time-lapse video microscopy for 90 minutes (pre addition). BDNF (10 ng/ml) or vehicle control was then added to the medium and migration of the same cells was followed for a second 90 minute period (post addition). (A) An individual *Bdnf*$^{-/-}$ granule cell was followed over time. The granule cell (arrow) remains stationary until BDNF is added then rapidly began to migrate along the glial cell. Arrow indicates the migrating granule cell, arrowhead indicates a fixed position within the images. (B) Cumulative data show that BDNF, but not vehicle control, increased the migratory index of granule cells and thus is acutely motogenic. We monitored the acute response of 212 cells to BDNF and 100 cells to vehicle control. $^{**}P<0.0006$ by χ^2 analysis.

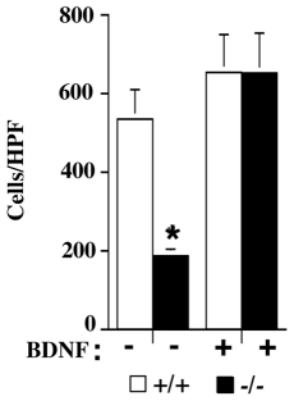

Fig. 6. Purified *Bdnf*$^{-/-}$ granule cells have defects in motility that are rescued by BDNF. Purified wild-type and *Bdnf*$^{-/-}$ granule cells were grown for 24 hours in Boyden chambers without (control) or with (+BDNF) 30 ng/ml BDNF in both chambers. Granule cells that migrated through the porous membrane into the lower chamber were directly quantified by light microscopy. Purified *Bdnf*$^{-/-}$ granule cells exhibited reduced migration in the control medium that could be rescued to wild-type levels by the addition of BDNF. **P*<0.05 (Student's *t*-test) for wild-type versus *Bdnf*$^{-/-}$ cells in control conditions. White bars, wild-type animals; black bars, *Bdnf*$^{-/-}$ animals. Pooled results from three separate experiments are shown.

above, granule cells were plated at high density and were already producing BDNF, potentially obscuring the effects of exogenous factor. Furthermore, while we and others (Lu et al., 2001) find that a uniform distribution of BDNF does not stimulate the movement of wild-type granule cells in Boyden chambers, a gradient of BDNF is able to induce directed movement of wild-type cells. This chemotactic effect would not have been appreciated in studies that analyzed a response to BDNF uniformly distributed in the solution. Here, using a variety of in vitro and in vivo assays to analyze both loss and gain of function, we demonstrate that BDNF stimulates the directed radial migration of granule cells.

Together the data presented here indicate that BDNF both stimulates migration of granule cells, and helps guide the migrating granule cells from the EGL to the IGL. These direct effects of BDNF on granule cell migration explain the layering defect of the BDNF mutant animals, in that the granule cells are impaired in their ability to initiate movement from the EGL to the IGL. Factors, such as Stromal cell derived factor, SDF (Ma et al., 1998; Klein et al., 2001), Ephrins (Lu et al., 2001) or MIA (Mason et al., 2001), are likely to work in concert with BDNF to regulate the radial migration of granule cells from the EGL to the IGL. We hypothesize that these other factors provide some redundancy, enabling the granule cells in *Bdnf*$^{-/-}$ mice to migrate eventually.

Since radial migration is a widespread phenomenon in the developing CNS, BDNF may have a general function in stimulating migration of early neuronal cells. In the cortex, BDNF, acting via the TrkB receptor, has been implicated as a chemotactic factor for early neurons (Behar et al., 1997), perhaps by regulating expression of reelin (Ringstedt et al., 1998). The related neurotrophin, NGF, can induce expression of cdk5 (Harada et al., 2001), a molecule critical for neuronal migration (Harada et al., 2001). However, these changes in gene expression are likely to be slow responses to neurotrophins. A novel finding here is the acute nature of the response to BDNF, suggesting that BDNF stimulates the migratory machinery, and does not merely regulate expression of proteins involved in migration. Future studies will be needed to determine if BDNF is required for radial migration of cortical neurons, in a manner analogous to the results shown here for cerebellar granule cells.

Neurotrophins activate two types of receptors, the Trk family of receptor tyrosine kinases and the p75 NTR (Kaplan and Miller, 2000). Stimulation of migration by BDNF apparently relies on activation of the TrkB receptors rather than the p75 NTR. The EGL is enlarged in animals with mutations of *Trkb* (*Ntrk2*) and *Trkc* (*Ntrk3*) (Minichello and Klein, 1996), but not in p75NTR mutant animals (A. R. C. and R. A. S., unpublished observations). Consistent with this interpretation, K252a, a pharmacological inhibitor of kinases including the Trks, hinders migration in Boyden chambers and in organotypic cultures (data not shown). In vivo, a gradient of BDNF may lead to the preferential activation of TrkB receptors located at the leading edge of the migrating granule cells, with lower levels of activated receptors at other subcellular locations.

The signaling pathways activated by Trk and responsible for neurotrophin-induced migration of granule cells have not yet been defined. However, studies examining the signaling pathways that enable NGF to function as a chemotactic agent for mast cells (Sawada et al., 2000), and numerous studies on cell migration in invertebrates suggest that activation of phosphatidylinositol 3 kinase or of small GTPases (Montell, 1999) are likely candidates. Differential localization of activated receptors and of critical signaling intermediates (Haugh et al.,

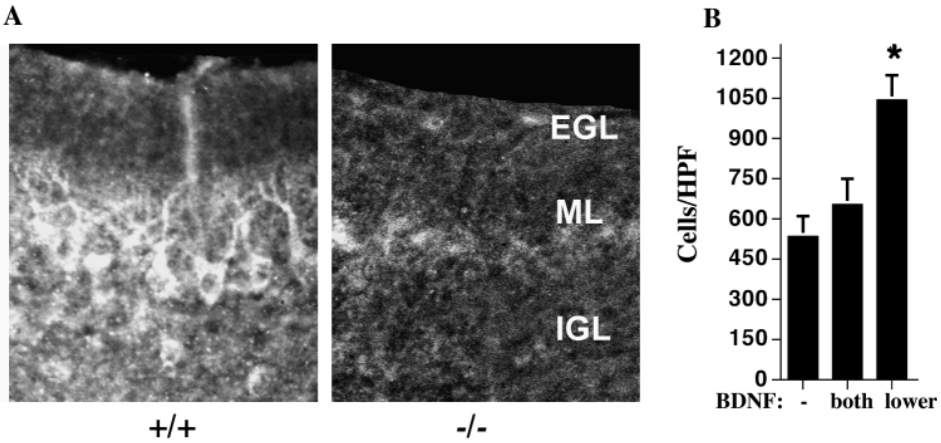

Fig. 7. Graded expression of BDNF may stimulate directed migration of granule cells. **(**A) Wild-type and *Bdnf*$^{-/-}$ cerebellar sections from P7 mice were immunostained with an antibody to BDNF. BDNF protein is present in a non-uniform distribution in the cerebellar cortex, with higher levels in the molecular layer and the IGL than in the EGL. (B) Purified wild-type granule cells were grown for 24 hours in Boyden chambers without BDNF (-), with 30 ng/ml BDNF added to both compartments (both) or with 30 ng/ml BDNF added only to the lower compartment (lower). As shown, a gradient of BDNF increased directed movement of wild-type granule cells through the porous membrane as compared to the movement with BDNF in both compartments, or to no BDNF. **P*<0.05 (Student's *t*-test) for control versus +BDNF, and for BDNF in both compartments versus BDNF in lower compartment only. Pooled results from three separate experiments are shown.

2000) within granule cells could provide a mechanism enabling BDNF both to stimulate migration and to provide a directional cue for movement.

This work was supported by grants from the NIH: P30-HD 18655, NS37757 (to R. A. S.); NS35884 (to G. C.); DA000522 and AI46999 (R. K. and A. L.), support from NARSAD and the EJLB Foundation (G. C.) and support from the Claudia Adams Barr Program (R. A. S.). P. R. B. was supported by a Freudenberger Fellowship, J. M. P. was supported by a fellowship from La Fondation pour la Recherche Médicale. In accordance with the Harvard conflict of interest guidelines, we note that R. A. S. has a financial interest in Curis, Inc. We thank Dr A. Welcher (Amgen, Thousand Oaks, CA) for the gift of recombinant BDNF. We thank Erin Berry and Hillary Gibson for technical assistance. We thank David Rowitch, Chuck Stiles and Scott Pomeroy for helpful discussions.

REFERENCES

Altman, J. and Bayer, S. A. (1997). Development of the Cerebellar System: In Relation to its Evolution Structure and Functions. New York: CRC Press.

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M. and Mikoshiba, K. (1994). A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. *J. Neurochem.* **63**, 1880-1890.

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V. M. and Mikoshiba, K. (1996). The mouse zic gene family. Homologues of the Drosophila pair-rule gene, odd-paired. *J. Biol. Chem.* **271**, 1043-1047.

Behar, T. N., Dugich-Djordjevic, M. M., Li, Y. X., Ma, W., Somogyi, R., Wen, X., Brown, E., Scott, C., McKay, R. D. and Barker, J. L. (1997). Neurotrophins stimulate chemotaxis of embryonic cortical neurons. *Eur. J. Neurosci.* **9**, 2561-2570.

Ernfors, P., Lee, K. F. and Jaenisch, R. (1994). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**, 147-150.

Fujita, S., Shimada, M. and Nakamura, T. (1966). ^{3}H-Thymidine autoradiographic studies on the cell proliferation and differentiation in the external and the internal granulear layers of the mouse cerebellum. *J. Comp. Neurol.* **128**, 191-208.

Gao, W., Zheng, J. and Karihaloo, M. (1995). Neurotrophin-4/5 (NT-4/5) and Brain Derived Neurotrophic factor (BDNF) act at later stages of cerebellar granule cell differentiation. *J. Neurosci.* **15**, 2656-2667.

Garcia-Zepeda, E. A., Combadiere, C., Rothenberg, M. E., Sarafi, M. N., Lavigne, F., Hamid, Q., Murphy, P. M. and Luster, A. D. (1996). Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *J. Immunol.* **157**, 5613-5626.

Goutan, E., Marti, E. and Ferrer, I. (1999). Expression of synaptic proteins in the developing rat cerebellum following ionizing radiation. *Int. J. Dev. Neurosci.* **17**, 275-283.

Harada, T., Morooka, T., Ogawa, S. and Nishida, E. (2001). ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat. Cell Biol.* **3**, 453-459.

Hatten, M. E. (1985). Neuronal regulation of astroglial morphology and proliferation in vitro. *J. Cell Biol.* **100**, 384-396.

Hatten, M. E. (1999). Central nervous system neuronal migration. *Annu. Rev. Neurosci.* **22**, 511-539.

Haugh, J. M., Codazzi, F., Teruel, M. and Meyer, T. (2000). Spatial sensing in fibroblasts mediated by 3′ phosphoinositides. *J. Cell Biol.* **151**, 1269-1280.

Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y. and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* **127**, 1185-1196.

Henderson, C. E. (1996). Role of neurotrophic factors in neuronal development. *Curr. Opin. Neurobiol.* **6**, 64-70.

Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. (1994). Targeted disruption of the bdnf gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989-999.

Kaplan, D. R. and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381-391.

Kenney, A. M. and Rowitch, D. H. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol. Cell Biol.* **20**, 9055-9067.

Klein, R., Martin-Zanca, D., Barbacid, M. and Parada, L. (1990). Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. *Development* **109**, 845-850.

Klein, R. S., Rubin, J. B., Gibson, H. D., DeHaan, E. N., Alvarez-Hernandez, X., Segal, R. A. and Luster, A. D. (2001). SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. *Development* **128**, 1971-1981.

Kobayashi, S., Isa, K., Hayashi, K., Inoue, H. K., Uyemura, K. and Shirao, T. (1995). K252a, a potent inhibitor of protein kinases, inhibits the migration of cerebellar granule cells in vitro. *Brain. Res. Dev. Brain. Res.* **90**, 122-128.

Lee, J. K., Cho, J. H., Hwang, W. S., Lee, Y. D., Reu, D. S. and Suh-Kim, H. (2000). Expression of neuroD/BETA2 in mitotic and postmitotic neuronal cells during the development of nervous system. *Dev. Dyn.* **217**, 361-367.

Lindholm, D., Dechant, G., Heisenberg, C. P. and Thoenen, H. (1993). Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity. *Eur. J. Neurosci.* **5**, 1455-1464.

Lu, Q., Sun, E. E., Klein, R. S. and Flanagan, J. G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. *Cell* **105**, 69-79.

Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T. and Springer, T. A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA* **95**, 9448-9453.

Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990). NT-3, BDNF and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* **5**, 501-509.

Mason, H. A., Ito, S. and Corfas, G. (2001). Extracellular signals that regulate the tangential migration of olfactory bulb neuronal precursors: inducers, inhibitors, and repellents. *J. Neurosci.* **21**, 7654-7663.

Minichello, L. and Klein, R. (1996). TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. *Genes Dev.* **10**, 2849-2858.

Miyata, T., Maeda, T. and Lee, J. E. (1999). NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. *Genes Dev.* **13**, 1647-1652.

Montell, D. J. (1999). The genetics of cell migration in Drosophila melanogaster and Caenorhabditis elegans development. *Development* **126**, 3035-3046.

Ringstedt, T., Linnarsson, S., Wagner, J., Lendahl, U., Kokaia, Z., Arenas, E., Ernfors, P. and Ibanez, C. F. (1998). BDNF regulates reelin expression and Cajal-Retzius cell development in the cerebral cortex. *Neuron* **21**, 305-315.

Rio, C., Rieff, H. I., Qi, P., Khurana, T. S. and Corfas, G. (1997). Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron* **19**, 39-50.

Rocamora, N., Garcia, L. F., Palacios, J. M. and Mengod, G. (1993). Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and low-affinity nerve growth factor receptor during the postnatal development of the rat cerebellar system. *Brain Res. Mol. Brain Res.* **17**, 1-8.

Sawada, J., Itakura, A., Tanaka, A., Furusaka, T. and Matsuda, H. (2000). Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Blood* **95**, 2052-2058.

Schwartz, P., Borghesani, P., Levy, R., Pomeroy, S. and Segal, R. (1997). Abnormal cerebellar development and foliation in BDNF$^{-/-}$ mice reveals a role for neurotrophins in CNS patterning. *Neuron* **19**, 269-281.

Segal, R., Pomeroy, S. and Stiles, C. (1995). Axonal growth and fasciculation linked to differential expression of BDNF and NT3 receptors in developing cerebellar granule cells. *J. Neurosci.* **15**, 4970-4981.

Segal, R. A., Takahashi, H. and McKay, R. D. (1992). Changes in neurotrophin responsiveness during the development of cerebellar granule neurons. *Neuron* **9**, 1041-1052.

Tanaka, S., Sekino, Y. and Shirao, T. (2000). The effects of neurotrophin-3 and brain-derived neurotrophic factor on cerebellar granule cell movement and neurite extension in vitro. *Neuroscience* **97**, 727-734.

Tapley, P., Lamballe, F. and Barbacid, M. (1992). K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene* **7**, 371-381.

Wetmore, C., Ernfors, P., Persson, H. and Olson, L. (1990). Localization of brain-dervived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. *Exp. Neurol.* **109**, 141-152.