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A rapid screening method for population-specific neuronal motogens, substrates and associated signaling pathways

Amani T. Hassoun¹, Ferenc Erdélyi², Gábor Szabó², and Margaret I. Davis^{1,*}

¹ Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892

² Departments of Gene Technology and Developmental Neurobiology, Institute of Experimental Medicine, H-1450, Budapest, Hungary

Abstract

We developed and characterized an assay that allows for rapid examination of migration of specific neuronal populations within a mixed population using the Boyden chamber principle. Migration of cerebellar interneurons and granule cells was examined using mice expressing enhanced Green Fluorescent Protein (eGFP) under the glutamate decarboxylase (GAD65) and growth-associated protein-43 (GAP43) promoters, respectively. Brain-Derived Neurotrophic Factor (BDNF) was used as the prototypic motogen for both populations. Fluorescent light-blocking inserts (FluoroBlok™) with different pore sizes and densities were compared in a 2 compartment assay. Immunodetection of polarity markers and nuclear staining indicated that dendrites and somata are preferentially extended through the pores in response to BDNF. Inserts coated with extracellular matrix (ECM) proteins were used to examine interactions between BDNF and the ECM during migration. ECM proteins alone stimulated migration when the lower side of the insert was coated, however coating of both sides of the insert slowed migration when compared to poly-D-lysine. Addition of a PI 3-kinase inhibitor to the lower compartment blocked BDNF-stimulated migration of both populations while a Src inhibitor reduced laminin-stimulated migration of interneurons, but not granule cells. We also examined use of neurons cultured from GAD65-eGFP mice as a reporter system for promoter activity. GAD65-eGFP mice may also be useful as a model for promoter regulation and the potential confounding effects of eGFP induction by the stimuli are also addressed. This assay allows for rapid analysis of motogens, substrates and signaling pathways that regulate migration of selected neuronal populations.

*Correspondence to: Margaret I. Davis, Ph.D., Section on Synaptic Pharmacology, Laboratory for Integrative Neuroscience, NIAAA/NIH, 5625 Fisher's Lane, MSC 9411, Bethesda, MD 20892, 301-443-4106, midavis@mail.nih.gov.
Amani T. Hassoun, B.S., Laboratory for Integrative Neuroscience / Section on Synaptic Pharmacology, National Institute on Alcohol Abuse and Alcoholism / National Institutes of Health, 5625 Fisher's lane, 2N-09, Bethesda, MD 20892, Telephone: 001-301-443-4108, Fax: 001-301-480-0466, Email: hassouna@mail.nih.gov
Ferenc Erdélyi, Ph.D., Transgenic Facility, Medical Gene Technology Unit, Inst. Experimental Medicine, HAS, Szigony str 43, Budapest H-1083, Hungary, Telephone: 00-36-1-210-9400, Fax: 36-1-2991002, Email: erdelyi@koki.hu
Gábor Szabó, M.D., Ph.D., Laboratory of Molecular Biology and Genetics, Inst. Experimental Medicine, HAS, Szigony str 43, Budapest H-1083, Hungary, Telephone 00-36-1-210-9400, Fax: 36-1-2991002, Email: szabog@koki.hu
Margaret I. Davis, Ph.D., Laboratory for Integrative Neuroscience / Section on Synaptic Pharmacology, National Institute on Alcohol Abuse and Alcoholism / National Institutes of Health, 5625 Fisher's lane, 2N-09, Bethesda, MD 20892, Telephone: 001-301-443-4108, Fax: 001-301-480-0466, Email: midavis@mail.nih.gov

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Keywords

migration; Boyden chamber; brain derived neurotrophic factor; NT-4; cerebellum; TrkB; interneurons; green fluorescent protein

1. Introduction

Process outgrowth and neuronal migration results from the integration of mechanical, cellular and chemical signals. Chemoattractants (motogens) initiate movement while haptotactic agents (the substrate, other cells and cell adhesion molecules) provide scaffolding on which the cells or processes move (Letrouneau, 1975; Milner and Campbell, 2002; Adams et al., 2002; reviewed by Hatten, 1999). The process of neuronal migration is targeted by multiple toxins and is frequently perturbed in spontaneously-occurring neurological mutants or by targeted deletions in transgenic mice (Heintz and Hatten, 2005). Therefore, a rapid method for examining the effects of mutations or toxins on neuronal migration would be useful for diverse areas of neuroscience research ranging from development and genetics to repair after injury or stroke.

Neuronal migration has been examined using a variety of techniques. Early studies relied on temporal histological examination (Fujita 1966; Palay and Chan-Palay, 1974; Bayer and Altman, 1975; Bayer et al., 1991; Altman and Bayer, 1997). Confocal and time-lapse microscopy have been employed to examine migration in the cerebellum *in situ* (Rakic and Komuro, 1995; Borghesani et al., 2002; Kumada et al., 2006) but this technique relies on local production of the motogenic and haptotactic factors and requires elaborate equipment with appropriate atmospheric and temperature control. In addition, repeated imaging of cells with a conventional scanning laser or UV light source can cause damage and contribute to oxidative stress; therefore migration can only be examined for short periods of time unless 2-photon excitation is used. Moreover, *in situ* identification of a small population of neurons, such as subpopulations of interneurons, is nearly impossible in a slice preparation without the addition of a genetic tag or selective label. Granule cell migration has also been examined *in vitro*, with directional and focal application of drugs and growth factors (Guan et al., 2007). Considerable knowledge has been gained using these techniques; however, none of these techniques are particularly suited for large-scale screening of substances, signals or substrates involved in migration.

The Boyden chamber assay (Boyden, 1962) was developed to examine leukocyte migration in response to chemotactic signals but has been used in other areas of biological research ranging from angiogenesis (Glasser et al., 1980) to neuronal migration (Hartmann et al., 1998; Klein et al., 2001; Borghesani et al., 2002). The Boyden chamber principle relies on two compartments separated by a porous barrier. Different pore sizes, densities, filter compositions, motogens and extracellular matrix components can be used to simulate the *in vivo* environment and cells can be coaxed by a stimulus to migrate through holes that are much smaller than the cell's nucleus. Pore sizes of 0.8 μm or less have also been used to selectively examine neurite outgrowth (Smit et al., 2003). The Boyden chamber has also been used to examine cerebellar granule cell migration since these cells represent approximately 90% of cerebellar neurons (Borghesani et al., 2002; Klein et al., 2001). The Boyden chamber technique works well with homogenous cell populations but becomes laborious when examining a mixed neuronal population over time since the identity of the cells would need to be determined by immunostaining or *a priori* by fluorescence-activated cell sorting.

We combined transgenic mice expressing Enhanced Green Fluorescent Protein (eGFP) under population-specific promoters with a modification of the Boyden chamber technique in order

to examine factors that regulate the postnatal migration of selected populations of cerebellar neurons. We examined TrkB-stimulated migration of granule cells expressing eGFP under the Growth-Associated Protein (GAP43) promoter and of GABAergic stellate and basket cells using mice expressing eGFP under the Glutamic Acid Decarboxylase (GAD₆₅) promoter. Cellular migration was analyzed using substrate-coated fluorescent light-blocking inserts (FluoroBlok™) in a double chamber 24-well array using conventional epifluorescent imaging and computer-assisted quantification. We evaluated the utility of this method for measures of matrix interactions through differential sensitivity of integrin-dependent versus integrin-independent migration to the Src family kinase inhibitor PP2. Finally, we examined the use of the eGFP transgene as a reporter for endogenous promoter activity and investigated this as a potential confounding variable in the migration assay. This assay can be adapted to analyze any neuronal population that can be selectively labeled with a fluorescent marker and allows for rapid screening of motogenic factors, substrates, toxins and for pharmacological analysis in a compartment-selective manner.

2. Methods

2.1 Mice

Transgenic mice (Tg(GAD65_3e/gfp3.3)#15) expressing eGFP under the GAD₆₅ promoter on C6CBA background were generated as described (Lopez-Bendito et al., 2004; Bali et al., 2005; Fiszman et al., 2005) and maintained by homozygous breeding. GAP43-eGFP mice on a Swiss-Webster background were generated using bacterial artificial chromosome technology (Gong et al., 2003, The Gene Expression Nervous System Atlas, GENSAT) and were obtained from the Mutant Mouse Resource Center (University of California, Davis). GAP43-eGFP mice were maintained by heterozygous breeding. We chose these strains because of the high expression levels in specific cerebellar populations, although other strains are available through GENSAT (www.gensat.org) or through individual investigators. All animal experimentation was performed in accordance with NIH guidelines for humane and ethical treatment of animals.

2.2 Histology and Fluorescence

Brains were removed from mice at postnatal day (PN) 7 and PN14. Brains were block fixed in 4% formaldehyde for 24 hr and a Vibratome® series 1000 was used to cut 50 µm parasagittal sections. Sections were counterstained with DAPI (0.5 µg/mL) for 20 min in Phosphate-Buffered Saline, 0.2% Triton X-100 (PBS-T), rinsed and dehydrated through graded ethanol. Sections were then hydrated through graded ethanol, rinsed in Tris-buffered saline (150 mM NaCl, 25 mM Tris, pH 7.4) and mounted in aqueous mounting medium. DAPI was imaged with 350/50 excitation and 460/50 emission filters but pseudo-colored red in some figures for better visualization in the presence of eGFP. EGFP was imaged using a 485/25 excitation filter and a 525/20 emission filter on a Zeiss Axiovert microscope with ACHROMAT 5X, NA 0.12 objective. High resolution images were collected using a Zeiss Pascal scanning laser confocal microscope with a Zeiss plan-neufluar 40X 1.3 NA objective. Images were collected at 1024×1024 resolution with 4 scan averages. Optical z-sections (20-30 at 1 µm steps) were taken through the tissue using the Ar-Kr 488 nm laser line. Laser intensity and gain were set such that the pinhole could be closed sufficiently for 1 µm resolution in the z-dimension. Projection images were generated from these z-series and are presented.

2.3 Migration Assay

2.3.1 Migration Chambers—Migration chambers were prepared using FluoroBlok™ inserts (Beckton-Dickinson, Franklin Lakes, NJ) and 24 well tissue culture plates (Costar, Corning, NY). Inserts were coated with either 100 µg/mL poly-D-lysine (polyK), 10 µg/mL fibronectin, 10 µg/mL laminin (all from Sigma, St. Louis, MO) or 25% growth factor reduced Matrigel™ (Beckton-Dickinson) for 2 hours or overnight. Extracellular matrix proteins and

polyK were diluted in water; Matrigel™ was diluted in Dulbecco's Modified Eagle's Medium (DMEM). Inserts coated with polyK were washed twice with sterile water prior to plating cells. Inserts were coated with 25 μ L of Matrigel™ spread to a thin layer with a cell scraper on the underside of the insert and the excess was aspirated. After the Matrigel™ solidified at 37°C, the upper chamber (when indicated) of the insert was coated and dispersed by tapping. Excess Matrigel™ was removed by aspiration. Inserts ranging from 1 μ m to 8 μ m pore sizes were tested. When substrates were varied with respect to the side of the barrier, inserts were first coated with polyK overnight followed by a 2 hr coating with the ECM protein (10 μ g/mL in water). Excess coating solution was aspirated and inserts allowed to dry. Longer incubation times with the substrate resulted in diffusion into the upper chamber. Preliminary experiments indicated that 10 μ g/mL for 2 hours was sufficient to promote adhesion and survival (Supplemental Fig.1). We chose to use 10 μ g/mL because this concentration supported adhesion but did not significantly increase survival when compared to polyK alone. Matrigel™ was used at 25% because cells clumped at lower concentrations and did not appear healthy.

2.3.2 Dissociation and plating of cells—Neonatal mice or rats (PN6-7) were euthanized with Fluorane®, cerebella removed and collected in Minimum Essential Medium on ice. Meninges and choroid plexus were removed and the tissue was minced and placed in 0.25% trypsin, 10 mM EDTA. Cells were dissociated for 20 min at 37°C in a shaking water bath. DNase (15000 U/mg, Sigma) was added to a final concentration of approximately 100 μ g/mL; cells were triturated with a pipette and incubated again for 5 min at 37°C. (Longer incubations with DNase were sometimes required when viscosity was too great for centrifugation.) Cells were centrifuged at 800g for 5 min and resuspended in DMEM (25 mM glucose, pyruvate), 2 mM glutamine, 1% fetal bovine serum, 2% B27 supplement, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ g/mL gentamicin (all from Invitrogen, Carlsbad, CA). Cells were diluted to 2.5 to 5 $\times 10^5$ in 300 μ L and this suspension was plated in the upper chamber. We examined the effect of cell density on migration (supplemental Fig. 2) and determined the optimal plating density to be between 2.5 to 5 $\times 10^5$ cells per well for both populations, although the higher end of this range was optimal for interneurons. Lower granule cell densities increased variability and higher densities caused an increase in background migration in the absence of a stimulus. However, due to litter size, it was sometimes necessary to decrease the number of cells plated to the lower end of this range. The optimal plating density should be determined for each population examined.

Plating medium (700 μ L) was added to the bottom chamber and cells were allowed to recover in the incubator at 37°C, 5% CO₂. After 2 hrs, the medium in the bottom chamber was replaced with the same medium formulation containing the motogen or inhibitor and motogen. DMSO (0.1%) was included in control and BDNF-stimulated wells for kinase inhibitor experiments to control for vehicle effects when necessary. When medium was replaced after 24 hrs, both upper and lower reservoirs were replaced. Migrating cells were examined by fluorescence microscopy over the first several hours or at 24 and 48 hr; 48 hr data are presented. Cells were also observed under halogen illumination prior to fluorescence imaging to verify the health of the cultures (white light is not blocked by FluoroBlok™). A movie showing initial steps of migration induced by BDNF in GAD-eGFP cells is shown in Supplemental Movie 1. Less than 10% of the total cells plated migrate over this time, probably due to the pore density.

2.3.3 Microscopy and Quantification—Inserts were imaged with a 10X, 0.3 NA Plan-NEO F objective on a Zeiss Axiovert 200 epifluorescence microscope with 485/25 excitation and 525/20 emission filters and standard 100W mercury source for quantification. Inserts in cell culture plates were also compatible with 32X LD and 40X LD ACHROPLAN objectives. The short working distance of conventional oil-immersion objectives prohibited high resolution imaging of inserts in the plate but the filters could be removed and imaged between coverslips (see section 2.4). Inserts were not compatible with Ar-Kr laser scanning confocal

microscopy. Images were captured using a 12-bit Zeiss Axiocam MR and Axiovision software using saturating exposure times determined from the intensity of the negative control. Images were converted to 8-bit TIFF files and migration was quantified using Image J (NIH Image; <http://rsb.info.nih.gov/ij/>). Images were thresholded to a value that allowed for detection of single particles (large leading processes and cell bodies) and all images within an experiment were thresholded to the same value. This insures that increases in process number or branching do not skew the analysis of migrating somata. A particle count was performed on the binary image and data are presented as mean number of particles normalized to the control (no stimulus) value. Average particle size after thresholding was also determined and is presented in some figures. For experiments examining the percent area covered by processes, images were converted to 16-bit TIFF files (Image J does not support 12-bit files) and similarly quantified using only background subtraction in order to retain the processes. These data are presented as area measurements. One-way analysis of variance was performed using Origin 7.5 (Microcal) and comparisons were considered statistically significant when p was less than 0.05.

2.4 Immunofluorescence

Inserts were fixed in 4% formaldehyde in PBS for 10 min. Filters were excised from the inserts with a scalpel and placed into 24 well trays. Filters were blocked for 1 hr with 5% Bovine Serum Albumin (BSA) in Phosphate-Buffered Saline, 0.2% Triton X-100 (PBS-T). Cells were incubated in primary antibodies for 1 hr, washed 3×10 min with PBS-T, and then incubated for 1 hr with Alexa 568 coupled secondary for 1 hr. Following 3 washes in PBS-T for 10 min, filters were mounted between 2 coverslips and imaged on both sides using a Zeiss Axiovert 200 epifluorescence microscope as described in 2.2. Primary rabbit anti-MAP2 (Chemicon, Temecula, CA) was used at 1:200, mouse B_{III} tubulin (Sigma) was used at 1:100 and chicken anti-neurofilament (EnCor, Alachua, FL) was used at 1:10,000. Secondary antibodies were obtained from Molecular Probes (Invitrogen). Alexa 568 anti-rabbit, anti-mouse or anti-chicken were used at 1:1000. EGFP and DAPI were imaged as above. Alexa 568 was imaged using 540/25 excitation and 605/55 emission filters. High resolution images of immunofluorescence were taken on excised inserts mounted between 2 coverslips and sealed with nail polish using a 100X 1.3 NA objective.

2.5 Viability Assays and eGFP levels

Cells were plated in the same medium used for migration assays on polyK-coated coverglass bottom chamber slides or 96 well plates and examined for total cell number, eGFP expression and 1-(4,5dimethyl-thiazol-2-yl)-3,5-diphenylformazan (MTT) conversion. Treatments were added after 2 hours at 2X (since medium could not be removed without disturbing the newly-plated cells) and assayed after 48 hr of treatment. eGFP-expressing cells were counted in 5 fields per chamber slide well from replicate wells using Image J as described above. Relative eGFP fluorescence was detected using cells lysed in 0.1% Triton X-100 containing a protease inhibitor cocktail (Sigma), 25 mM Tris, pH 8.0 and measured using FITC excitation and emission filters on a Genios Plus spectrofluorometer and Magellan data acquisition software using the automated signal amplification mode (TECAN, Männedorf/Zurich, Switzerland). Specificity of the signal was validated by examining the excitation and emission spectra using a SpectraMAX Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA); there was no contamination of the eGFP signal since both excitation and emission spectra were consistent with purified eGFP (Lippincott-Schwartz and Patterson, 2003). Replicate wells were processed for MTT conversion as previously described (Mosman, 1983). For MTT assays, medium was removed after 48 hr and replaced with complete growth medium containing 0.5 mg/mL MTT. Cells were incubated for 2 hours, medium aspirated and the formazan product was dissolved in isopropyl alcohol. Absorbance was measured at 550 nm using a SpectraMAX Plus

microplate spectrophotometer (Molecular Devices). Data were normalized to control values and analyzed as described above.

3. Results

3.1 *In Vivo* Distribution

The development of the cerebellum is well-established, particularly with respect to granule cell and Purkinje cell birth and migration. Figure 1 shows the distribution of eGFP in GAD₆₅-eGFP and GAP43-eGFP strains at PN7 and PN14; these time points correspond to the peak and end of the major migratory wave for both granule cells (Fujita et al., 1966) and GABAergic basket and stellate cells (Yamanaka et al., 2004; Zhang and Goldman 1996). The DAPI counterstain (pseudo-colored red) primarily shows granule cells since they are the densest population in the neonatal cerebellum.

GAD₆₅ is expressed by Purkinje cells (Greif et al., 1991) but eGFP is not detected at significant levels Purkinje cells in the GAD₆₅-eGFP mice. The GAD₆₅-eGFP transgene is expressed most robustly in stellate and basket cells in the cerebellum. GABAergic basket and stellate cells migrate from their birth place in the white matter to the molecular layer beginning during the first postnatal week (Fig. 1A; Zhang and Goldman, 1996; Yamanaka et al., 2004). The birth and migration of the interneurons occurs heterogeneously with lobule-selective peaks, as shown by the arrow in figure 1A for lobule VIII and IX at PN7. At the end of the migratory period (PN14), most of the interneurons populate the molecular layer, with basket cells residing closer to the Purkinje cell layer and stellate cells present in the outer molecular layer (Ramon y Cajal, 1911; Yamanaka et al., 2004 and Fig. 1B). In contrast, GAP43-eGFP-expressing granule cells are born in the EGL and migrate in the opposite direction over the same time course (Fig. 1C). This is more readily appreciated by the dense nuclear staining (Fig. 1C compared to Fig. 1D, white arrow denotes the EGL) since eGFP is most apparent in the processes of these cells. eGFP expression is most robust in the leading processes of the granule cells at PN7 (Fig. 1C, red arrow). The majority of the granule cell soma is occupied by its nucleus, thus little eGFP is visible in the cell body at this resolution but granule cell axons (parallel fibers) are apparent in the molecular layer at PN14 (Fig. 1D, red arrow). High resolution confocal images of the interneurons (1E) and granule cells (1F) are also shown. Factors that regulate the migration of granule cells have recently been identified and include BDNF (Borghesani et al., 2002) and stromal-derived factor (Klein et al., 2001). There is little known about factors regulating the migration of the interneurons, however.

3.2 Migration Assay

3.2.1 Configuration and Validation—A schematic of the chamber configuration is shown in Fig. 2A. The inserts are coated with substrate on both sides or on the bottom only, as indicated. Cells are plated in the top reservoir and allowed to adhere and recover from the dissociation process. After 2 hours, the bottom chamber is filled with medium identical to that in the top chamber, with or without the motogenic stimulus or DMSO as a control, when appropriate. FluoroBlok™ inserts block excitation of the upper chamber, allowing for examination of neurites and migrating cells over time in the lower chamber with epifluorescence microscopy. Cells above the insert are visible under halogen illumination, which allows for examination of cell viability and adhesion. Various pore sizes and densities can be employed and the pores are shown under low level halogen illumination in Fig. 2B. To determine the molecular permeability of the 1 μm pores we examined the diffusion of recombinant purified Venus, which is of similar size to the motogens examined (approx. 28 kDa). Approximately 14% of the input Venus protein (25 nM in PBS added to PolyK coated inserts) was recovered in the upper chamber after 28 hours of incubation at 37°C when measured spectrofluorometrically (Fig. 2C). Very little protein diffused into the upper chamber

at the 1-6 hr time points. There was significant protein binding to the cationic substrate since we could recover Venus protein from the inserts with SDS extraction (not shown).

When a motogen (BDNF or NT4) is added to the bottom chamber, there is typically a 5-15 fold increase in the number cell bodies and neurites in the lower chamber that is maximal at 48-72 hrs, as depicted in Fig. 2D for granule cells. Note the large soma-like particles and numerous processes. We chose to terminate the assay at 48 hrs because of the dependence of granule cells on depolarization for prolonged survival (Balazs et al., 1988; D'Mello et al., 2003) and due the complexity of the processes with longer incubation times. Image J was used to quantify particles on the lower side of the inserts. Thresholded 8-bit images are shown in Fig. 2E (GAP43-eGFP). In order to verify that the large particles counted represent nuclear translocation and that GFP is not interfering with migration (see Agbulut et al., 2007, for example), we stained the inserts with DAPI to detect nuclei using rat granule cells (thresholded nuclei are shown in Fig. 2F). A higher power of the images is shown in Fig. 2G with the thresholded areas shown in red. Approximately 10% of the particles counted are large dendritic processes, most likely cells in the early stages of migration.

Granule cells are known to migrate in response to BDNF and it is a potent motogen in this assay, stimulating migration in cells from GAP43-eGFP mice by up to 14-fold with a plateau at 10 ng/mL (Fig. 2H). NT4, another TrkB ligand, also stimulated granule cell migration, although this was less robust at the 10 ng/mL concentration and slightly higher, but not significantly so, at the 50 ng/mL concentration (Fig 2H). BDNF also robustly stimulated GABAergic interneuron migration in cells from GAD₆₅-eGFP mice by over 6-fold with a plateau also at 10 ng/mL (Fig. 2I). NT4 stimulated interneuron migration by 3.5 fold but, in contrast to granule cells, did not reach the level of BDNF-stimulated migration at 50 ng/mL. Quantification of BDNF-stimulated migration of rat granule cell nuclei is shown in Fig. 2J. The fold increase is greater when measuring thresholded particles than when measuring nuclei. This is because nucleokinesis is incomplete at 48 hours, but can be increased with longer incubation times or larger pore sizes. Many cells extended a large bleb through the pore that is the size of a cell body but have not completed nucleokinesis. This somatic structure is similar to what has been described in migrating granule cells where the nucleus trails the somatic tubulin cage (Solecki et al., 2004)

The particles quantified above primarily represent neurons in the process of migration, but neurotrophins also induce process outgrowth. In order to determine whether this method could be used to measure processes, we converted the 12-bit images to 16-bit images (Image J does not support 12-bit) and measured the area covered by GFP in background-subtracted images. The average particle count for these experiments for granule cells and interneurons are shown in Fig 3 A and B, respectively. Representative thresholded images for granule cells (Control, Fig. 3C and BDNF, Fig. 3D) and interneurons (Control, Fig. 3E and BDNF, Fig. 3F) are shown for comparison. While the particle count in these experiments was increased 5-10 fold, depending on the cell type, the percent area increased by greater than 20-fold in granule cells and up to 8-fold in interneurons in the presence of BDNF (Figs. 3 G,I). These area values were then divided by the raw values obtained using 8-bit, highly thresholded images (primarily somatic, as shown in Fig 2E) to obtain the average area per particle (Figs. 3 H,J). These data are confounded by neurotrophins regulation of the GAD₆₅ promoter (see below) and should therefore be interpreted with caution in this line of mice. These data suggest that BDNF is a more potent motogen for both cell types but that NT-4 may increase process outgrowth to a greater extent than BDNF. Similar to BDNF-stimulated migration, NT-4 stimulated process formation shows signs of desensitization at 50 ng/mL. This difference in concentration response and desensitization is suggestive of differential potencies of BDNF and NT-4 in mediating cellular responses despite sharing a common receptor.

FluoroBlok™ inserts are available in 3 pore sizes of 1, 3 and 8 μm and the pore density varies within the pore sizes tested ($1.6 \times 10^6/\text{cm}^2$ for 1 μm , $0.8 \times 10^5/\text{cm}^2$ for 3 μm and $10^5/\text{cm}^2$ for 8 μm). While both cell populations will migrate through all of the pore sizes examined, the fold increase of particles counted was greatest using the 1 μm size (Fig. 4 A, C). We were unable to measure changes in particle number in response to BDNF using the 8 μm pore size using interneurons, however more complete somatic migration and clumping (an average thresholded particle size of 15 μm when stimulated with BDNF) occurred in the BDNF treated 8 μm chambers (Fig. 4B). Granule cells are much smaller than the interneurons (approximately 5-6 μm in diameter) and they fell through the 8 μm pores so migration was subsequently measured using the smaller pore sizes (Fig. 4C). As with the interneurons assayed with 8 μm pores, granule cells did not show a significant increase in particle count when assayed using 3 μm pores (Fig. 4C) but we did detect an increase in particle size in response to BDNF (Fig. 4D). This assay configuration has a gravity component that is apparent with the larger pore size. We chose to use the 1 μm pore size for the majority of our assays because the density of the pores is twice that of the 3 μm size and the size of the pores was nearly optimal for both cell types. When examining a small subpopulation in a mixed population, the use of a higher pore density increases the likelihood that a cell will be close to the source of the stimulus. This is substantiated by the observation that the fold increase in the number of particles counted is proportional to the difference in pore density between the 2 pore sizes (Fig. 4A). It should also be noted that nucleokinesis occurs more readily at 48 with the 3 μm pores. The ideal pore size for granule cells is closer to 2 μm , however, these inserts are not produced with pores of this size.

3.2.3 Identity of Migrating Processes—Neurons follow defined migratory patterns that are population-specific. For example, granule cells extend a leading process and follow it along Bergmann glial fibers while leaving a trailing process, the axon or parallel fiber, behind (Rivas and Hatten, 1995). Polarity is also regulated by growth factors which can stimulate both dendrite and axon formation. We removed the filters from the insert cartridge with a scalpel and stained them for markers of neuronal polarity to determine whether BDNF was stimulating dendritic or axonal growth. The top of the insert is shown in Fig. 5A and E for interneurons and granule cells, respectively. MAP2 staining is shown in red and DAPI (blue) was used to visualize the nuclei; the migrating particles in both populations were readily identified as somato-dendritic by their expression of MAP2 (Fig. 5 B, C, F, G) and Tuj1 (not shown). The correlation between DAPI staining and pore size indicates that the particles counted are leading processes and somata from migrating neurons. Comparison of eGFP expression to MAP2 immunostaining in Fig. 5 A-C shows that the majority of the migrating cells from the GAD₆₅- eGFP mice do not express eGFP and are mostly granule cells. This can also be appreciated by the rough concordance between eGFP and MAP2 immunofluorescence in GAP43-eGFP expressing granule cells (Fig. 5 E-G), although a significant amount of the eGFP signal was lost during immunostaining. We did not observe staining for neurofilament, a marker of axons, on either side of the insert after 48 hours, and glial fibrillary acidic protein-expressing glial cells were not detected at a significant level in the lower chamber. Both granule cells and interneurons are able to migrate completely through both 1 and 3 μm filters but more nuclei are visible with the 3 μm filters (compare blue channel in Fig. 5 B, F to Fig. 4 C,G). The continuum of the migratory process observed between the different pore sizes indicates that the cells are in the process of somatic translocation (migration) but less complete nucleokinesis occurs with the 1 μm inserts. High-power images of these fully migrated nuclei through 1 μm inserts are shown for interneurons (Fig. 5D) and granule cells (Fig. 5H). The image obtained under halogen light illumination is also shown, revealing the cells on the other side of the insert for comparison, channels are shown separately in Supplemental Figure 2.

3.2.4 Comparison of Substrates—Neurons use neurophilic, gliophilic and extracellular matrix factors such as laminin and proteoglycans for migration (Hatten, 1999). Polycationic amino acids are used to promote neuronal adhesion *in vitro*, but this is primarily through electrostatic interactions with cell surface proteoglycans. ECM molecules such as laminin and fibronectin engage transmembrane receptors, integrins, to produce more selective adhesion. These ECM proteins are also capable of outside-in signal transduction through focal adhesion kinase-like proteins that signal to Src, Rac, Rho, MAP kinases, and PI 3-kinase, among others, to regulate adhesion and cytoskeletal dynamics (Schlaepfer and Hunter, 1998). We coated the inserts on both sides with polyK, laminin, fibronectin, or growth factor reduced Matrigel™ (25%) to examine interactions between the substrate coating and BDNF-stimulated migration. In this configuration, all ECM components delayed migration when compared to polyK in both cell populations (Fig. 5 A,B). It should be noted that Matrigel™ solidifies and clogs the pores, therefore cells must digest the matrix in order to migrate. When we incubated the interneurons for 72 hours, we observed significant migration (Supplemental Fig. 3) indicating that cells will digest a matrix in response to a BDNF gradient.

Adhesion can also be prohibitive for migration (Lauffenburger and Horwitz, 1996; Gupton and Waterman-Storer, 2006), therefore we hypothesized that the cells simply adhered too well to the substrate on the top of the insert to migrate. Recent evidence also suggests that integrin-mediated signaling can desensitize growth factor signaling (Galownia et al., 2007). Images of the cells on the top of the ECM-coated inserts are shown in supplemental Fig 1. ECM components supported more granule cell viability, as determined by DAPI staining, and the cells were uniformly adhered to all substrates except for cellular fibronectin, which caused cells to clump slightly but did alter cell viability. Since cellular fibronectin was second to polyK in supporting migration, these data suggest inhibition of BDNF-stimulated migration by the ECM. We observed significant migration when the top of the insert was coated with polyK and the bottom with ECM proteins (Fig. 6 C, D) indicating that ECM proteins alone are a signal for migration. Interneuron migration stimulated by BDNF (10 ng/mL) was not additive with the ECM proteins examined with the exception of plasma fibronectin (Fig. 6C). In contrast, migration of granule cells was increased by BDNF regardless of the substrate but did not show additivity (Fig. 6D). Processes on laminin were longer and straighter than on polyK; representative images of BDNF-stimulated granule cell process outgrowth on polyK (Fig. 6E) and laminin (Fig. 6F) are shown for comparison.

3.2.5 Signaling Pathways Mediating Migration—Migration depends on intracellular signal transduction pathways, although different cell populations use different biochemical pathways. It is also apparent that the subcellular location of the signaling intermediates determines the response (Heerssen and Segal, 2002). We examined the role of 2 well-conserved signaling pathways, PI 3-kinases and Src-family kinases, in BDNF-stimulated migration on polyK-coated inserts. Inclusion of the PI 3-kinase inhibitor LY 294002 (50 μ M) in the lower chamber inhibited migration of both cell populations (Fig. 7 A, B). We also assayed neuron survival by MTT conversion at 48 hrs in the upper chamber when 50 μ M LY 294002 was included with BDNF in the lower chamber (Fig. 7C). This treatment reduced viability by 35%. Therefore inhibition of migration by LY 294002 may be partially do to toxicity even when applied selectively to leading processes. The Src family kinase inhibitor PP2 (10 μ M) did not inhibit migration of either cell type on polyK (Fig. 7 A, B).

Adhesion to polyK is primarily thought to be electrostatic and integrin-independent, therefore we performed the same assay on inserts coated with laminin on the bottom only. As previously shown in Fig. 6, laminin alone stimulated migration (Fig. 7 D, E) and this is blocked by PP2. BDNF still induced significant migration that was partially inhibited by PP2, but this was only significantly different in the interneurons. This degree of inhibition by PP2 is roughly equivalent to the enhanced migration observed when the bottom of the chamber is coated,

indicating that BDNF stimulates migration independent of Src regardless of the substrate. Laminin-stimulated and BDNF-stimulated migration appear to be additive with granule cells but not with interneurons suggesting these 2 populations use different extracellular and intracellular pathways for migration while responding to the same motogen. Furthermore, the different appearance of the processes under the 2 conditions (Fig. 6 E,F) indicates that the cytoskeletal dynamics may differ as well depending on the substrate.

3.3.1 Regulation of the GAD₆₅ promoter by TrkB ligands—BDNF is known to upregulate the GAD promoter (Arenas et al., 1996) and to increase survival of granule cells (Segal et al., 1992). The effect of BDNF on cerebellar interneuron survival is not well-established, however Fisman and colleagues (2005) reported increased spine size and increased survival of stellate and basket cells with BDNF in long-term cultures. An increase in cell survival is a potential variable to control for so we used 3 methods to address this issue. First, we plated cells in standard 96-well tissue culture plates and treated them with increasing concentrations of BDNF and NT4. The cells were lysed and eGFP fluorescence was measured in a microplate spectrofluorometer (Fig. 8A, B). BDNF increased eGFP levels in the GAD₆₅-eGFP cells by 65% (Fig. 8A) but only increased eGFP levels in GAP43-eGFP granule cells by 15-20% (Fig. 6B). To determine whether GAD₆₅-driven eGFP expression was due to an increase in cell number, we counted eGFP⁺ cells in cultures from GAD₆₅-eGFP mice and found no differences between treatment groups but an increase in eGFP fluorescence when sister cultures were lysed and analyzed with a spectrofluorometer (Fig. 8C). We were not able to detect differences in intensity on images from the microscope on a per cell basis (not shown). Finally, we used MTT conversion to measure survival in the cultures as a whole and found that BDNF increased survival by ~25% (Fig. 8C). This increase represents an increase in granule cell survival since there was no difference in the number of eGFP⁺ interneurons and correlates with the observed increase in eGFP fluorescence in BDNF and NT4 treated GAP43-eGFP cultures (Fig. 7B). We also measured eGFP fluorescence in the top chamber to determine whether application of the neurotrophin to the dendritic compartment was capable of increasing eGFP levels in the upper chamber. BDNF or NT4 in the lower chamber signals to GABAergic cell bodies in the upper chamber since we also observed an increase in eGFP fluorescence in the top chamber (Fig. 7D). This may reflect diffusion of BDNF into the upper chamber or may result from transport of BDNF back to somata still on the other side of the membrane.

This observation does not invalidate our measurements since we are unable to detect the difference with epifluorescence and quantification was performed on binary images. The thresholded image does not reflect differences in intensity, but theoretically the size of the particles could vary when thresholded. We did not observe this phenomenon but this may become apparent if the promoter is strongly regulated by the stimulus. This is easily controlled for by staining with a general membrane stain such as DiI for comparison with the eGFP signal. Granule cell viability was increased by 15-25%, depending on the assay used, in the presence of BDNF. The increase in migration is much greater therefore it is not likely to be due to increased granule cell survival but increased survival may contribute a small amount to the migration signal observed.

4. Discussion

The use of transgenic mouse technology has opened new avenues for research but also requires that established protocols be adapted and expanded to maximize the use of this technology. We have modified the classical Boyden chamber assay to take advantage of transgenic mice expressing eGFP in selected neuronal populations. Using the cerebellum as a model, we show stimulation of migration and neurite outgrowth in granule cells and GABAergic interneurons with TrkB ligands. This represents motility of dendrite-like processes since they are immunopositive for dendritic markers. NT-4 and BDNF are potent motogens for both postnatal

migratory populations in the cerebellum, although they migrate in opposite directions over the same period in development. The concentration of BDNF that supported migration in granule cells is in agreement with our previous studies on BDNF-stimulation of ERK in granule cells, which also reached a maximum between 5 and 10 ng/mL (Ohrtman et al., 2006). We also examined the effect of extracellular matrix proteins on BDNF-stimulated migration and showed that integrin engagement slows migrations when compared to polyK but varying substrate location may be useful for some experiments. Varying substrate coatings is also useful in distinguishing integrin-mediated vs. integrin-independent events. This assay provides a rapid screen for population-specific neuronal motogens that allows for examination of migration over time. Similar strains of eGFP-expressing mice can also be crossed with transgenic or mutant mice, thereby increasing the utility of the assay for large-scale analysis.

Multiple lines of eGFP-expressing mice are available from GENSAT or through individual investigators but this assay requires relatively high per cell eGFP expression levels. This insures that all processes are counted, regardless of minor changes in expression level. Because the inserts sit well above the optics in the configuration we employed, the assay is limited by the working distance of the objective. Low power objectives are ideal because the captured field is larger but higher power objectives such as those with correction collars or long working distance (LD) objectives are also suitable. The configuration we used is not compatible with oil-immersion imaging but the inserts can be removed and imaged between glass coverslips. Microplate spectrofluorometers can also be used to increase throughput of this assay; however, this method of quantification is more subject to absolute levels of eGFP, slit width of the instrument relative to process size, and does not allow for quantification of particle area or size.

Examination of eGFP levels in microplate assays and in the chamber assay indicates that some eGFP expressing mouse lines may also be useful for measuring promoter activity. Interestingly, the eGFP signal did not plateau at 50 ng/mL concentration of BDNF in granule cells whereas migration and ERK activation do. This could be the result of high eGFP stability or may represent differential regulation of migration and GAD₆₅ promoter activity. We did not observe significant accumulation of BDNF-stimulated eGFP at 24 hrs, suggesting the signal needs time to accumulate. In addition, the time required to appropriately fold eGFP into a fluorescent conformation may limit the usefulness of this approach for rapidly regulated genes. The observation that addition of TrkB ligands to the lower compartment can increase eGFP expression driven by the GAD₆₅ promoter in the upper chamber is particularly interesting. This suggests that the signal, or more likely the neurotrophin itself, might be transported intracellularly across the barrier. Further experiments will be required to determine the contribution of transport and diffusion to this effect.

The effect of the motogen or stimulus on the promoter driving eGFP expression should also be considered as a potential confounding variable. The GAD₆₅ promoter showed regulation by BDNF in our system but the GAP43 promoter did not, correlating instead with an increase in overall survival. A plausible explanation lies within the regulation of expression of these proteins. GAD₆₅ is readily regulated at the level of mRNA expression (Qin et al., 1994) whereas GAP43 is regulated at the level of mRNA stability and translation (Perrone-Bizzozero et al., 1993). The 3' regulatory sequences that confer this translational regulation are absent from the GAP43-eGFP construct since it only contains the 5' regulatory sequences. Because the eGFP levels were extremely high in the GAD₆₅-eGFP neurons and the images were thresholded and converted to binary images for quantification, BDNF-stimulated eGFP expression likely did not alter our analysis of migration. The amount of eGFP detected using a spectrofluorometer was increased significantly but we did not detect any change in cell number with BDNF treatment. These data indicate that the amount of eGFP is increasing on a per cell basis but we do not detect this by epifluorescent microscopy through the plastic used in cell culture wells.

It may be possible to measure eGFP levels with well-calibrated optics and multiple high-resolution images. The eGFP signal is diluted through multiple processes and may require 3-dimensional volume and intensity measurements to be quantifiable by fluorescence microscopy.

Many of the drugs used in signal transduction experiments are toxic to developing neurons and we examined whether inclusion of these compounds in the bottom chamber would kill the neurons in the top chamber. The amount of the compound that is able to diffuse through the pores is small and charge-dependent with polyK-coated inserts. Even if the drug is predicted to be diluted to a non-toxic level, delivery of a toxin to the neurites may influence the soma on the other side of the barrier. Previous studies have indicated that LY 294002 is toxic *in vitro* (Davis et al., 2003) and we observed cell death in the upper chamber when LY 294002 (50 μ M) was included in the bottom chamber. Toxicity can be controlled for at the termination of the assay by performing a viability assay such as MTT conversion or propidium iodide uptake for apoptosis on the cells in the top chamber. This aspect of the chamber makes it ideal for studies of compartmentalization, particularly with larger cells only capable of extending processes through the 1 μ m pores. In addition, inserts that do not block fluorescence are available with smaller pore sizes and could be adapted for similar studies. The preferential extension of dendrites into the bottom chamber also may be useful for studies of neuronal polarity.

We observed the greatest amount of motility when cells were assayed using poly-D-lysine-coated inserts. The cells are better adhered to laminin or fibronectin than polyK, which can slow migration (Lauffenburger and Horwitz, 1996; Gupton and Waterman-Storer, 2006). When the bottom of the insert was coated with laminin, we observed an increase in unstimulated migration that was inhibited by PP2. Conversely, BDNF-stimulated migration on polyK was insensitive to PP2. This observation is not surprising since polyK is frequently used as a negative control in assays of integrin signaling (Schlaepfer et al., 1998, for example), and argues against matrix molecules from serum binding to polyK as a mechanism for attachment. Varied substrate placement and differential sensitivity to inhibitors also suggests that different signaling pathways are active in neurotrophin-stimulated versus integrin-mediated adhesion. These experiments show that BDNF-stimulated migration is independent of matrix and but can be modulated (slowed or directed) by ECM signals.

We have characterized a modification of the Boyden chamber assay with utility in diverse areas of neuroscience research. The use of fluorescent light blocking inserts, various ECM proteins and eGFP expressing mice allows for examination of multiple aspects of neuronal migration in a temporal and population-specific manner. By comparing migration of granule cells and interneurons from the cerebellum, we determined that TrkB ligands stimulate migration of both populations. These data suggest that the directional cues are provided by extracellular signals, cells or substrates *in vitro*. Furthermore, inclusion of pharmacological inhibitors of intracellular signaling pathways suggests that there are subtle differences in the absolute dependence on Src for migration between the two neuronal populations. This assay will be useful in further characterizing the signal transduction pathways used by specific neuronal populations and the biochemical interactions between these pathways and substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. GABAergic interneurons migrate from the white matter into the molecular layer while granule cells migrate from the EGL into the IGL during the second postnatal week in the mouse. DAPI was used to stain nuclei in vibratome sections (50 μ m) of the neonatal cerebellum at the beginning (PN7) and end (PN14) of the migratory peak in GAD₆₅-eGFP (A, B) and GAP43-eGFP (C, D) mice. The arrow in A and B denotes the stellate and basket cells as they move past the Purkinje cell layer and into the molecular layer (compare A to B). The white arrows (B, C) show the EGFP which is still present at PN14. EGFP is apparent in the processes of migrating granule cells at PN7 (red arrow, C) but shifts to the molecular layer granule cell

axons upon the completion of migration (D, red arrow). Also note the dramatic thickening of the IGL at PN14 (D, *).

Fig. 2.

A schematic diagram of the modified Boyden chamber is shown in (A). FluoroBlok™ inserts are coated with a substrate and placed in a cell culture well. The motogen is added to the bottom chamber and the cells are imaged from the bottom. Various pore sizes and densities were employed and are shown in (B). One micron pores were only slightly permeable to Venus over a 28 hr period (C, n=3). A representative micrograph of neurite outgrowth and somatic migration of GAD-eGFP neurons through 1 μm pores is shown in (D). Representative thresholded binary images are shown in E (GAP43-eGFP) and F (DAPI stain of granule cell nuclei). Higher resolution of these cells and the thresholded image (red) are shown in (G). BDNF and NT4 were used as prototypic motogens and both stimulated migration of GAP43-

eGFP neurons (H, n=6) and GAD₆₅-eGFP neurons (I, n=12). Quantification of DAPI stained nuclei is shown in (J) for comparison. Data represent the mean $\pm$ SD (C) and mean $\pm$ SE (E,F). *, $p < 0.05$ when compared to control by ANOVA.

Fig. 3.

Quantification of neurite outgrowth stimulated by BDNF and NT4 using 16-bit images compared to 8-bit images. Thresholded 8-bit particle counts are shown in A and B for granule cells and interneurons, respectively. Representative background-subtracted images are shown in C and D (granule cells, control and BDNF-stimulated) and E and F (interneurons, control and BDNF-stimulated). The normalized total area and area per particle is shown in G and H (granule cells) and I and J (interneurons), respectively. Data represent the mean $\pm$ SE of 8-12 replicates. *, $p < 0.05$ when compared to control by ANOVA.

Fig. 4.

Comparison of pore size and fold increase in migrating particles with GAD₆₅-eGFP (A, n=3) and GAP43-eGFP (B, n=3) neurons. The fold increase in migration observed with the higher density 1 μ m pores is greater than with the 3 μ m pores and no BDNF-stimulated increase in particle count was observed with 8 μ m pores. The difference in fold migration between the 2 pore sizes reflects the higher density of pores on the 1 μ m inserts. Cells from GAD₆₅-eGFP were stimulated with 50 ng/mL BDNF and GAP43-eGFP cells were treated with 10 ng/mL BDNF. Data represent the mean $\pm$ SD of 3 replicates.

Fig. 5.

Immunofluorescence detection of dendritic markers in the lower chamber of the insert. Cells from GAD₆₅-eGFP mice were stimulated with 50 ng/mL BDNF and GAP43-eGFP mouse cells were stimulated with 10 ng/mL BDNF. Plated cells on the top of the filter are shown in A (interneurons) and E (granule cells). Somata and dendrites stained for MAP2 (red) are abundant whether plated on 1 μ m (B,F) or 3 μ m (C,G) inserts. Interneurons are shown A-D and granule cells are shown in E-H. DAPI staining (blue) for nuclei indicates that more nucleokinesis occurs with the 3 μ m pores. Higher power images (D,H) show the cells above the insert examined under white light illumination and migrating cells while nuclei (DAPI) on the bottom of the insert were imaged by epifluorescence.

Fig. 6.

Comparison of substrates and location of ECM proteins. Migration was evaluated using inserts coated on both sides with poly-D-lysine, cellular fibronectin (cFN), plasma fibronectin (pFN), laminin (LN) (all at 10 μ g/mL) and 25% Matrigel™ after 48 hours of stimulation with BDNF. Cells from GAD₆₅-eGFP were stimulated with 50 ng/mL BDNF and GAP43-eGFP cells were stimulated with 10 ng/mL BDNF. Extracellular matrix proteins present on both sides of the insert significantly slowed migration when compared to poly D-lysine (A, interneurons, B, granule cells, n=4). When the top was coated with polyK and the bottom with the ECM, the substrate alone supported migration when compared to polyK and this was increased by BDNF

for both interneurons (C, n=3) and granule cells (D, n=3) depending on the substrate. * $p < 0.05$ compared to polyK control, + $p < 0.05$ compared to individual control coating.

Fig. 7.

PI 3-kinase and Src family kinase inhibition differentially effects migration depending on the substrate. PI 3-kinase inhibition with LY 294002 (50 μ M), but not Src inhibition with PP2 (10 μ M), blocked interneuron (A, n=8) and granule cell (B, n=6) migration on polyK. PP2 did not decrease migration of either population when assayed on polyK. When the top of the insert was coated with polyK and the bottom with laminin, PP2 blocked migration on laminin and reduced the BDNF-stimulated response in interneurons (C, n=12) but not significantly in granule cells (D, n=9). Addition of LY 294002 to the lower chamber decreased viability of cells in the upper chamber as measure by MTT conversion (E). * $p < 0.05$ compared to control, # $p < 0.05$ compared to LN-BDNF, + $p < 0.05$ compared to LN-BDNF+PP2, NS = not significant.

** p<0.05 compared to BDNF, # p<0.05 compared to laminin, + p<0.05 compared to BDNF on laminin.

Fig. 8.

Both BDNF and NT4 increase expression of eGFP from the GAD₆₅ promoter. Cells were treated for 48 hrs in 96 well plates, lysed and eGFP was measured in a spectrofluorometer (A,B and the first 2 columns in C, n=8). Both BDNF and NT4 robustly increased eGFP expression in interneurons (A) but not in granule cells (B). Cell counts indicate that the increase in eGFP is not due to an increase in cell number at 48 hrs (C, n=20, 5 fields taken in each quadrant and the center of 4 chamber slide wells). MTT conversion performed in a 96-well plate indicates that granule cell viability is increased by BDNF (C, last 2 columns, n=8). This increase in MTT conversion by granule cells correlates with the increase in eGFP levels observed in B. BDNF and NT4 added to the lower chamber increased eGFP levels measured with a

spectrofluorometer in lysates of interneurons in the upper chamber (D, n=4). * $p < 0.05$ compared to control, ** $p < 0.05$ compared to BDNF. Data represent the mean $\pm$ SE.