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THE EFFECTS OF NEUROTROPHIN-3 AND BRAIN-DERIVED NEUROTROPHIC FACTOR ON CEREBELLAR GRANULE CELL MOVEMENT AND NEURITE EXTENSION *IN VITRO*

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Abstract—Migration of the granule cells is a major stage of cerebellar maturation. Granule cells express neurotrophins and their receptors; however, their role in cell migration has not been defined. In this study we investigated the effects of exogenous neurotrophins on the movement and neurite extension of granule cells from glial-free cerebellar cell reaggregates *in vitro*. Our results provide direct evidence that neurotrophin-3 and brain-derived neurotrophic factor differentially affect the granule cells. Neurotrophin-3 significantly affected granule cell movements by decreasing the migration index (the ratio of the number of cells that moved further than half the neurite length) and the speed of cell soma movement, but did not affect neurite length or growth cone migration. In contrast, brain-derived neurotrophic factor and neurotrophin-4 acted on growing neurites and growth cones by significantly increasing neurite length and the speed of growth cone migration, but had no effect either on the migration index or on the speed of the cell soma movement.

The results suggest that neurotrophins differentially affect neurite extension and the movements of cerebellar granule cells.

Key words: cerebellar granule cell culture, neuronal migration, neurite extension, NT-3, BDNF.

The structure and connectivity of the mature nervous system is the result of neuronal migration and the extension and withdrawal of neurites, and the complex molecular events that underlie these developmental processes are now under investigation. In the developing cerebellum, one of the key stages is the migration of the granule cells. Neurotrophins and their receptors are present in the developing cerebellum,[20] suggesting that they may play a role in cerebellar development. Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are considered to be survival factors of granule cells,[4,14,18,23,29] and BDNF is known to promote neurite extension.[1,14,19,31] However, the effect of neurotrophins on cell migration is unclear. NT-3 knockout mice exhibit no morphological defects in the cerebellum,[9,10,37–39] suggesting that NT-3 does not affect cell migration. In contrast, other studies using hypothyroid rats which have a deficit in NT-3[27] and rats implanted Elvax, which offers exogenous NT-3,[7] suggest that NT-3 promotes cell migration. Therefore, it remains unclear whether NT-3 has any effects on cell migration or not. The experiments described in this paper are directed at understanding the actions of neurotrophins on cerebellar granule cell migration. These *in vitro* studies take advantage of the tendency for dispersed cerebellar cells to reaggregate under certain conditions, allowing us to study the movements of cells and the extension of neurites under defined conditions. Portions of these studies have been presented in abstract form.[36]

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Abbreviations: BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle medium; GFAP, glial fibrillary acidic protein; MAb, monoclonal antibody; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; PBS, phosphate-buffered saline; PLL, poly-L-lysine hydrobromide; Trk, tyrosine kinase receptor.

EXPERIMENTAL PROCEDURES

Chemicals

Insulin–transferrin–sodium selenite, putrescine, poly-L-lysine hydrobromide (PLL) and laminin (purified from mouse EHS sarcoma) were obtained from Sigma (St Louis, MO, U.S.A.). Dispase II and DNase I were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The neurotrophins human nerve growth factor (NGF), BDNF, NT-3 and neurotrophin-4 (NT-4) were obtained from Pepro-Tech EC Ltd. (London, U.K.). Monoclonal antibody (MAb) against glial fibrillary acidic protein (GFAP) was obtained from Boehringer Mannheim. Cell proliferation was studied by bromodeoxyuridine (BrdU) uptake and demonstrated immunocytochemically using a kit from Amersham International (Buckinghamshire, U.K.). Other cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.).

Cell culture

All experiments were carried out according to the Animal Care and Experimentation Committee, Gunma University, Showa Campus. All efforts were made to minimize animal suffering and reduce the number of animals used. Cerebellar granule cells were cultured according to the method of Kobayashi *et al.*[17] Cells were cultured on glass coverslips coated with PLL and laminin. Coverslips (18 or 24 mm in diameter) were sterilized, immersed in PLL (100 μg/ml overnight; 4°C) followed by laminin (500 ng/ml; 2 h at 37°C). Immediately before use, the coverslips were rinsed with Dulbecco's modified Eagle medium (DMEM).

Granule cells were obtained from two-day-old male Wistar rats. Rats were anesthetized with ether and the brains rapidly dissected; the cerebellum was digested with Dispase II and DNase I, and triturated. Dissociated cells from three to five cerebella were suspended in Ca^{2+}–Mg^{2+}-free Hanks' balanced salt solution, centrifuged (5 min, 800*g*) and resuspended at a concentration of 1×10^6 cells/ml in DMEM (with 10% fetal bovine serum). To allow the reaggregates to form, the cell suspension was incubated at 37°C for 12 h (5% CO_2/95% air) and centrifuged (8 min, 800*g*). The cells were cultured in

serum-free medium consisting of DMEM and Ham's F-12 medium (1:1), supplemented with glucose (6 mg/ml), $NaHCO_3$ (450 mg/ml), insulin (25 mg/ml), transferrin (25 mg/ml), sodium selenite (25 mg/ml), progesterone (6.3 ng/ml) and putrescine (9.7 mg/ml). Aliquots (100 μl) of the reaggregate suspension were plated on the prepared coverslips (18 mm). The coverslips were placed in tissue culture dishes and incubated for 30 min to allow the reaggregates to adhere to the laminin substrate. The cells were then cultured for up to 36 h in serum-free media with or without neurotrophins (NGF, BDNF, NT-3, NT-4; 20 ng/ml each).

Immunocytochemistry

To investigate the characteristics of the cells, the cultures were stained with MAb against drebrin (M2F6[33]) or GFAP. Cultures were fixed in 4% paraformaldehyde (30 min), washed with phosphate-buffered saline (PBS), permeabilized (0.1% Triton X-100 in PBS, 10 min) and washed in PBS followed by bovine serum albumin (3% in PBS, 30 min). Cultures were sequentially incubated with primary antibody (1:1 M2F6, 1:500 GFAP; 2 h) and fluorescein-conjugated antibody against mouse immunoglobulin G antibody (1:100; 2 h) at room temperature with PBS rinses in between. The coverslips were mounted on glass slides with PermaFluor (Immunon, Pittsburgh, PA, U.S.A.). Specimens were observed under a fluorescence microscope equipped with ×40 Nomarski differential interference contrast (DIC) optics (Axioplan; Zeiss).

Quantification of neurite length and migration index

Typical reaggregates (100–200 μm in diameter) were randomly selected from five to 10 cultures; 10 reaggregates from each culture were photographed under phase-contrast (Diaphoto TMD 300; Nikon) with ×10 optics and with ×10 or ×40 Hoffman module optics (Hoffman modulation contrast; Modulation Optics Inc.). The field of each photograph included the tip of the longest neurite and the edges of the reaggregate. To calculate the neurite length per reaggregate, we measured the length of the longest neurite from the margin of the reaggregate to the tip of the neurite, then repeated the measure on nine nearby neurites.

Cell movement was quantified by determining a "migration index", as described previously.[17] For this measure, arcs were drawn from the margin of the reaggregate with a radius equal to one half of the neurite length. Then two radial lines were drawn from the margin of the reaggregate. These two radial lines were set at an angle of about 60° so as to count more than 100 cells between these two lines (Fig. 2A). The "migration index" was calculated using the following equation: migration index (%) = (the number of cells beyond the arcs between two radial lines)/(the total number of cells between two radial lines) × 100.

Analysis of mitotic activity

Uptake of the thymidine analog BrdU was used to determine cell proliferation[26] using a kit from Amersham International according to the manufacturer's protocol. Cells were incubated with BrdU (3 μg/ml) for 3 h and fixed in acid–ethanol (90% ethanol/5% acetic acid/5% water, 30 min). For immunocytochemistry, cultures were incubated sequentially with an MAb against BrdU (1:100; 1 h) followed by anti-mouse immunoglobulin G–peroxidase conjugate and developed in 3,3′-diaminobenzidine tetrahydrochloride. The BrdU labeling index was calculated using the following equation: BrdU labeling index (%) = (the number of BrdU-positive cells moving away from the aggregates)/(the total number of cells moving away from the aggregates) × 100.

Time-lapse video microscopic analysis

Cultures were observed using a time-lapse DIC video microscopy system (XL-10; Olympus). This allowed us to continuously monitor the behavior of individual cells and neurites. Reaggregates were plated on 24-mm laminin-coated coverslips; the coverslips were placed in a Teflon dish, which was inserted into a 37°C tissue culture chamber on the microscope with Nomarski DIC optics. The images were recorded with a video camera (C2741; Hamamatsu Phonics) and were stored in an optical memory disk recorder (LVR-3000AN; Sony) every 2 min. To enhance the contrast of cell somata and growth cones, the video gain was increased while an offset voltage was applied; this increased the range of brightness levels between black and white and decreased the background gray level. All the images included the center of the reaggregate and the growth cones of the longest neurites.

To determine the trajectory of movement of individual cell somata and growth cones, we selected images of 10 typical cells and neurites; we then superimposed the traces of these cell somata and growth cones recorded over 1 h. To calculate the speed of movement, we determined the movement distances of individual cell somata and growth cones during a 10-min recording time.

Statistical analysis

All statistical analyses were performed by using StatView for Macintosh 4.5 statistical software. All data are expressed as the mean ± S.E.M. and analysed by ANOVA followed by *post hoc* Fisher tests. A difference between test and control responses was considered significant with a P value <0.05.

RESULTS

Neurite extension and cell movement in reaggregation cultures of rat cerebellar granule cells

The radial extension of cell processes and the radial movement of neuron-like small cells from the reaggregates were observed within 5 min of plating in serum-free medium. The extent of neurite extension and cell movement from the reaggregates with time in culture are shown in Fig. 1A and B. To determine if the neurites and cells were neuronal or glial, the reaggregates were reacted for the neuron-specific antigen drebrin (Fig. 1D), using MAb M2F6, and the glial antigen GFAP (Fig. 1C). The neurites and moving cells were positive for drebrin; the only GFAP-positive cells were found inside the reaggregate (data not shown). Furthermore, cells apparently moving away from the reaggregates had a spindle-shaped and bipolar morphology, similar to that described for granule cells.[2] We concluded that the moving cells and extending processes from the reaggregates were most likely cerebellar granule cells, rather than glial, stellate or basket cells.

Effects of neurotrophins on the neurite length and migration index of cells

To determine if BDNF and NT-3 affected the proliferation of cells in the reaggregation cell cultures, we assessed DNA synthesis by BrdU uptake. BDNF and NT-3 had no effect on the BrdU labeling index for cells moving away from the aggregates (Table 1). As shown in Table 1, the BrdU labeling indices for all treatment groups were the same: 33–36% at 12 h, 23–30% at 24 h and 18–22% at 36 h. Thus, BDNF and NT-3 do not affect the BrdU labeling index during the 36-h culture period. A previous study[14] demonstrated that neurotrophins did not affect the BrdU labeling index of granule cells in the reaggregates.

The effects of neurotrophins on neurite extension were analysed from images taken at 12, 24 and 36 h of culture (Table 2A). At 36 h, BDNF and NT-4 significantly increased neurite length by 12–14% compared with controls. In contrast, NGF and NT-3 had no effect on neurite length. None of the neurotrophins affected the morphology of the neurites, i.e. branching or fasciculation.

We then studied the effects of neurotrophins on cell movement. Cultures grown in NT-3 showed an inhibition of cell movement (Fig. 2D) compared to those grown in control medium (Fig. 2A) or medium containing either NGF (Fig. 2B) or BDNF (Fig. 2C). Cell movement was quantified by calculating the migration index for each condition. This index

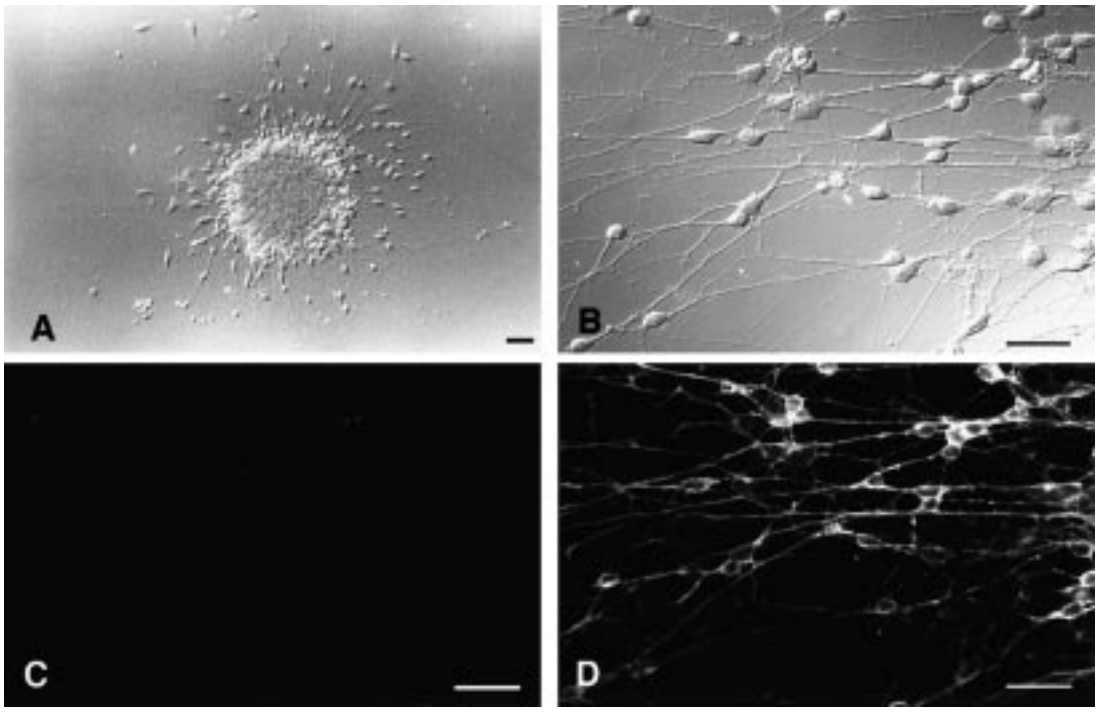

Fig. 1. Characterization of neurites and moving cells from reaggregates. Reaggregates of cerebellar granule cells from two-day-old rat cerebella were plated on glass coverslips coated with PLL/laminin and cultured in serum-free media. (A) A Hoffman Modulation contrast image of a reaggregate, with extending neurites and moving cells, after 12 h of incubation. (B–D) Neurites and moving cells after 24 h of incubation. B and D are Nomarski DIC and fluorescence images of a culture immunostained with an antibody against drebrin. C is a fluorescence image of a similar culture to B, immunostained with antibody against GFAP. Note that extending neurites and migrating cells are immunonegative for GFAP, while they are immunopositive for drebrin. Scale bars = 50 μm.

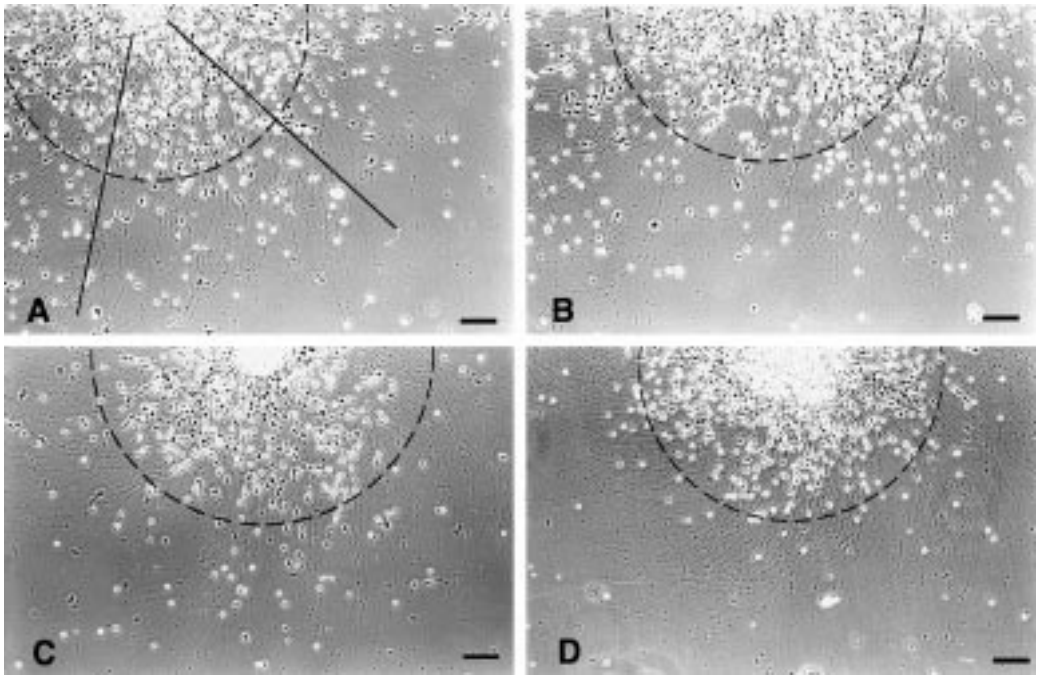

Fig. 2. Effects of neurotrophins on neurite extension and cell movement in reaggregation cell cultures after 36 h of incubation. (A–D) Phase-contrast images of reaggregates cultured in serum-free media without exogenous neurotrophins (control; A), with NGF (B), with BDNF (C) and with NT-3 (D). The arcs represent half the neurite length from the margin of the reaggregates. The migration index is the ratio of the number of cells between two radial lines beyond these arcs compared to the total number of cells between two radial lines. Note that treatment with NT-3 markedly inhibits granule cell movement. Scale bars = 50 μm.

allows us to compare counts from reaggregates of different sizes, thereby giving us suggestive data on cell movement.[17] In control medium, the migration indices did not vary significantly with time in culture. Only NT-3 exposure, for 36 h, significantly decreased the migration index (Table 2B).

Promotion of growth cone motility by brain-derived neurotrophic factor: time-lapse video microscopic analysis

Using a time-lapse video microscopy system, we charted the behavior of growth cones and calculated their growth

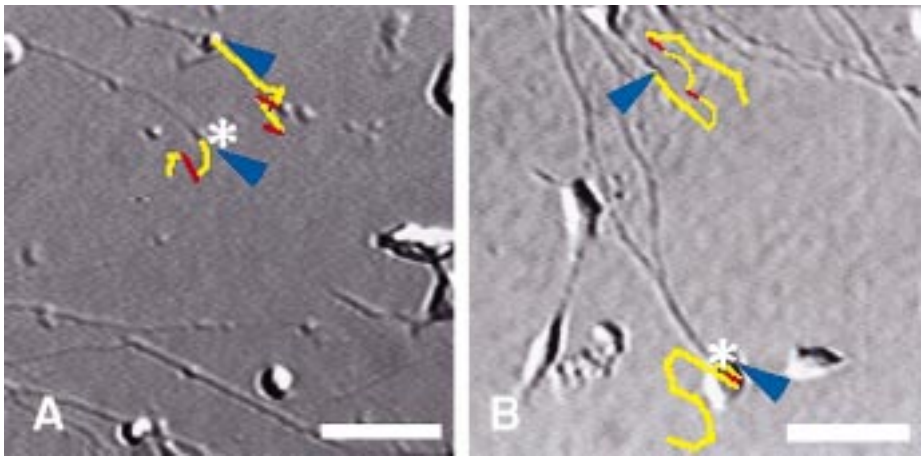

Fig. 3. Time-lapse characterization of neurite extension in reaggregation cell cultures. The positions of growth cones were plotted every 2 min for the period between 24 and 25 h of incubation. Superimposed traces of the trajectories of growth cone movement are shown. (A) In this control reaggregate, the growth cones move away from the reaggregate (growth cone migration; yellow lines), and most growth cones move as if they were changing their direction. Their movement is not smooth or continuous, and sometimes they move backwards (neurite retraction; red lines). Blue arrowheads indicate starting points. (B) A reaggregate treated with BDNF. Note that treatment with BDNF increases the growth cone migration distances. Asterisks indicate typical growth cones, which are described in the text. Scale bars = 50 μm.

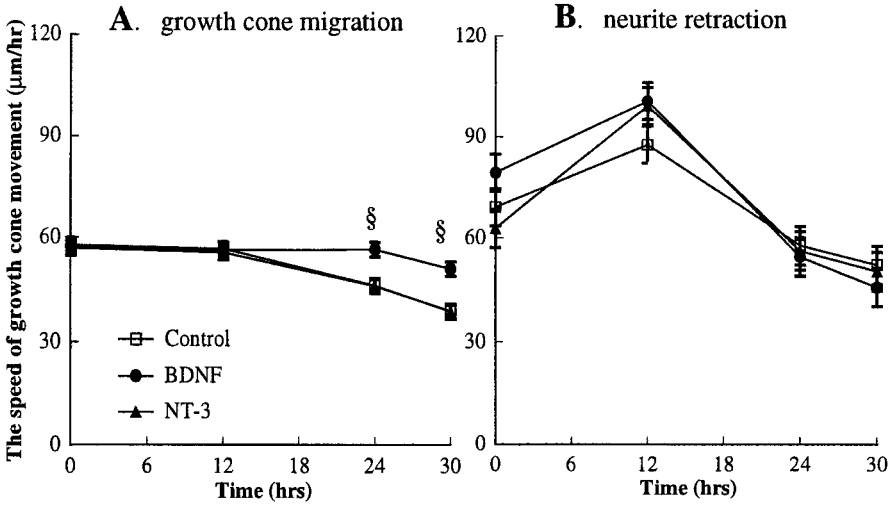

Fig. 4. BDNF promotes growth cone migration in reaggregation cell cultures. The graphs show the mean speed of growth cone migration (A) and neurite retraction (B) after 0, 12, 24 and 30 h of incubation without exogenous neurotrophins (open squares), with BDNF (filled circles) and with NT-3 (filled triangles). A shows that BDNF significantly increases the speed of growth cone migration at 24 and 30 h ($n = 31–51$, mean ± S.E.M.; §$P < 0.05$), while B shows that these neurotrophins have no significant effect on the speed of neurite retraction.

Table 1. The effect of neurotrophins on cell proliferation *in vitro*

	Percentage of cells positive for BrdU with time in culture		
	12 h	24 h	36 h
Control	33.1 ± 1.4	29.7 ± 2.0	21.0 ± 1.0
BDNF	34.4 ± 1.6	28.6 ± 1.7	21.8 ± 1.1
NT-3	35.1 ± 1.8	23.5 ± 3.2	18.4 ± 1.5

The BrdU labeling index is calculated as the percentage of cells that were stained with anti-BrdU antibody. Treatments with 20 ng/ml BDNF and NT-3 have no significant effects on either index. Results are mean ± S.E.M.

speed. Growth cone movement was characterized as either "growth cone migration" (away from reaggregate; yellow lines in Fig. 3) or "neurite retraction" (causing rapid backward movement of growth cone; red lines in Fig. 3). In the majority of neurites, the growth cone moved away from the reaggregate. If neurites of growth cones moving toward the reaggregate were positioned on the opposite side of the cells from the reaggregate, this type of growth cone movement was regarded as growth cone migration. BDNF affected growth cone migration but not neurite retraction. BDNF did not affect the frequency of growth cone migration (data not shown), but significantly increased the distance of growth cone migration. For example, the total distance of typical growth cone migration from 24 to 25 h in control media (Fig. 3A) was 36.4 μm, whereas that in BDNF-supplemented media (Fig. 3B) was 69.1 μm. BDNF had no effect on the distance of neurite retraction; NT-3 had no effect on growth cone migration or neurite retraction.

We next studied the speed of growth cone migration. The speed of growth cone migration in control media decreased with time in culture (Fig. 4A, open squares). BDNF significantly increased the speed of growth cone migration at 24 and 30 h (Fig. 4A, filled circles). NT-3 had no effect on the speed of growth cone migration (Fig. 4A, filled triangles). The speed

Table 2. The effect of neurotrophins on neurite length and migration index

	12 h	24 h	36 h
(A) Neurite length (μm)			
Control	30.8 ± 2.6	62.3 ± 1.3	74.9 ± 2.6
NGF	33.2 ± 1.5	61.9 ± 1.8	73.7 ± 1.3
BDNF	32.0 ± 2.3	63.8 ± 2.1	84.9 ± 1.6*
NT-3	31.0 ± 2.6	61.6 ± 1.4	73.2 ± 1.9
NT-4	28.9 ± 1.6	63.7 ± 1.4	83.9 ± 1.8*
(B) Migration indices (%)			
Control	25.4 ± 2.1	25.1 ± 1.0	25.8 ± 1.5
NGF	25.8 ± 2.3	25.4 ± 1.0	25.1 ± 1.3
BDNF	26.8 ± 2.4	24.6 ± 1.7	26.7 ± 2.0
NT-3	24.6 ± 1.5	20.9 ± 1.9	17.3 ± 1.9*
NT-4	25.5 ± 1.6	24.6 ± 1.2	26.8 ± 2.0

Neurite lengths and migration indices after incubation for 12, 24 and 36 h with and without neurotrophins ($n = 10$). (A) BDNF and NT-4 significantly increased the neurite length at 36 h. (B) NT-3 significantly decreased the migration index at 36 h. Results are mean ± S.E.M. *$P < 0.005$.

of neurite retraction in control medium also decreased with time in culture (Fig. 4B, open squares). Neither BDNF nor NT-3 affected the speed of neurite retraction (Fig. 4B).

Inhibition of granule cell motility by neurotrophin-3: time-lapse video microscopic analysis

The yellow lines in Fig. 5 show the superimposed traces depicting the trajectory of representative moving cells between 24 and 25 h after plating in control and NT-3-supplemented media. Neither BDNF nor NT-3 changed these movement patterns of cells or the shapes of cell somata.

NT-3 decreased the speed of cell soma movement. For example, a typical cell in control medium (Fig. 5A) moved 54.2 μm in 1 h, whereas a typical cell in NT-3-supplemented medium (Fig. 5B) moved 27.6 μm. We compared the mean speed of cell soma movement for cells grown either in control or NT-3-containing medium ($n = 30$, three experiments). Cells in control media moved at 60.3 ± 3.8 μm/h (0 h) and at 61.0 ± 4.3 μm/h (12 h); this speed gradually decreased to 43.9 ± 3.3 μm/h (24 h) and 28.3 ± 3.9 μm/h (30 h) (Fig. 6, open squares). NT-3 significantly decreased the mean speed of cell soma movement to 17.1 ± 2.9 and 8.5 ± 1.6 μm/h at 24 and 30 h, respectively (Fig. 6, filled triangles). BDNF did not affect the speed of cell soma movement at any time (Fig. 6, filled circles).

DISCUSSION

In this paper, we have addressed the issue of the role of neurotrophins in granule cell migration. Using a glial-free culture system, and careful measurement of neurite and cell soma movements, we have shown that BDNF promotes neurite extension and NT-3 inhibits cell movement. For this work we modified the reaggregation cell culture technique so that we could measure cell movement,[3,13] as well as neurite extension and cell proliferation,[1,13,14,31] by using laminin-coated coverslips.[17] Using similarly modified reaggregation cell cultures, Bix and Clark[5] reported that stimulation of platelet-activating factor receptor inhibits granule cell migration.

One of the problems in studying neurons in culture is the problem of glial cell contamination. In the developing cerebellum, drebrin is highly expressed only by early postnatal cerebellar granule cells, but not by glial cells.[6,32,33] In our cultures, we were able to demonstrate that drebrin, but not GFAP, was present in all of the moving cells and processes extending from the reaggregates. Using immunocytochemistry, Bix and Clark have also showed that migration of granule cells from reaggregate involved the extension of processes along other pre-extended neurites (neuronal class

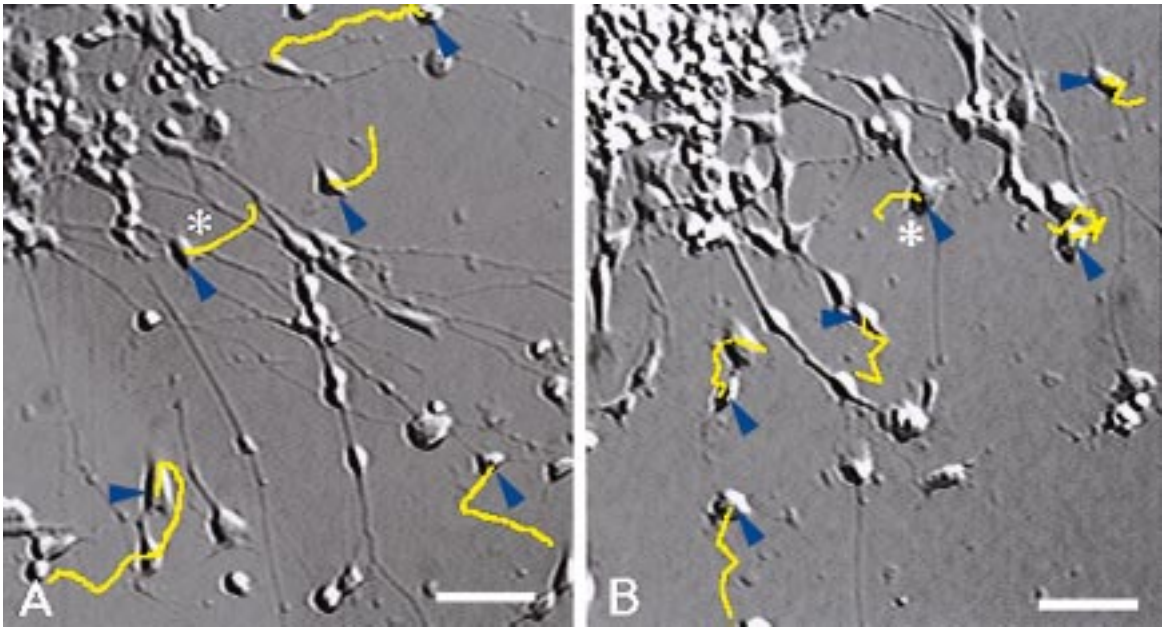

Fig. 5. Time-lapse characterization of granule cell movement in reaggregation cell cultures. The positions of cell somata were plotted every 2 min for the period between 24 and 25 h of incubation, and the superimposed traces of the trajectories of cell movement are shown (yellow lines). Blue arrowheads indicate starting points. (A) Control reaggregate. These cells moved away from the reaggregate, although they occasionally paused briefly or moved backwards. (B) A reaggregate treated with NT-3. Note that NT-3 decreases the cell movement distances, but does not change the movement pattern of cells. Asterisks indicate typical cell somata, which are described in the text. Scale bars = 50 μm.

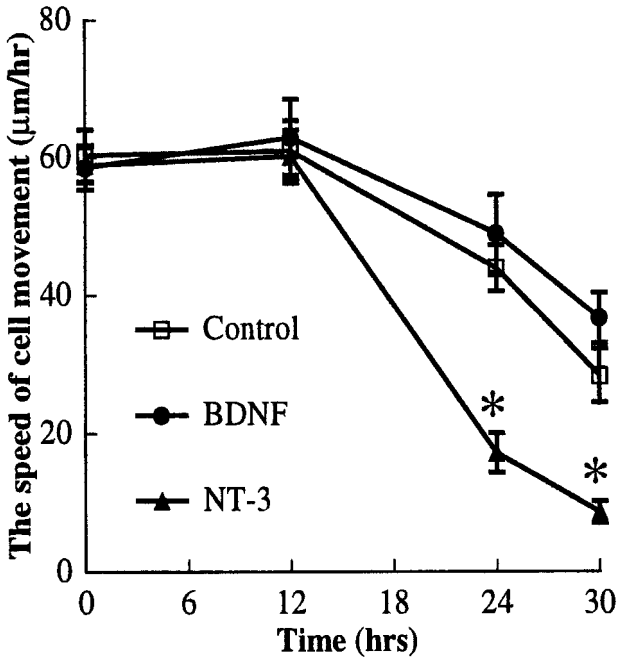

Fig. 6. NT-3 inhibits cerebellar granule cell movement in reaggregation cell cultures. The graph shows the mean speed of cell soma movement after 0, 12, 24 and 30 h of incubation without exogenous neurotrophins (open squares), with BDNF (filled circles) and with NT-3 (filled triangles). NT-3 significantly decreased the speed of cell soma movement at 24 and 30 h ($n = 30$, mean ± S.E.M.; $^*P < 0.005$).

III β-tubulin-specific marker; TUJ-1 positive, GFAP negative).[5] Furthermore, the majority of moving cells had the typical spindle-shaped and bipolar morphology characteristic of granule cells. We concluded that the moving cells and their processes outside of the reaggregates were granule cells and their neurites. This predominantly glial-free environment allowed us to analyse the effects of neurotrophins on granule cell movement and neurite extension directly without the confounding influence of the surrounding glia.

Brain-derived neurotrophic factor promotes neurite extension in vitro

Our data showed that BDNF and NT-4, but not NT-3, significantly increased neurite length, which is consistent with other studies.[14,31] Neurites extend with forward migration of growth cones, and occasionally retract with rapid backward movement of growth cones.[8,25] We therefore studied the effects of BDNF and NT-3 on the behavior of the growth cones.

Neither BDNF nor NT-3 affected the frequencies of growth cone migration and neurite retraction (data not shown). However, BDNF increased the speed of growth cone migration but had no effect on the speed of neurite retraction. NT-4 resembles BDNF in its specific neuronal action and in its ability to specifically activate the TrkB tyrosine kinase receptor.[15,16] Therefore, it is possible that the increased neurite length produced by BDNF and NT-4 results from promoting the growth cone migration in reaggregation cell cultures. In contrast, NT-3 had no effect on growth cone migration.

In our studies, BDNF exposure for 24 h significantly increased the speed of growth cone migration and increased neurite length. This time frame is in agreement with reports by Segal *et al.*[31] using similar cultures. These data suggest that BDNF and NT-4 do not stimulate the initial extension of neurites, but that these neurotrophins act either to enhance neurite extension or to stabilize growing neurites. Members of the immunoglobulin superfamily, and components of the extracellular matrix, are potential targets for the effects of neurotrophins, since they influence granule cell extension and fasciculation.[12,21,40] BDNF and NT-4 have been shown to increase the level of mRNA for growth-associated protein-43 within 48 h.[31] It will be interesting to determine if growth-associated protein-43 itself affects the speed of growth cone migration.

Neurotrophin-3 inhibits cerebellar granule cell migration in vitro

The role of NT-3 in cell migration is controversial. In this study, NT-3 decreased the speed of cell soma movement and the migration index, but did not affect neurite growth. Studies using NT-3 knockout mice indicated that NT-3 does not affect granule cell migration;[9,10,37–39] however, studies using organotypic cultures,[30] hypothyroid rats[27] and Elvax implants[7] have supported a role for NT-3 in promoting granule cell maturation and migration. There are several possible explanations for the discrepancies between our findings and those from the previous studies.

One possibility is the difference in cell migration pathways between cells in our studies and in previous studies. In the developing cerebellum, granule cells first migrate medially and laterally in the transverse plane of the deep external granular layer; this migration is independent of glial cell processes (tangential cell migration).[28] Then, four days after birth, the cells migrate radially from the external granular layer to the internal granular layer along Bergmann glial cell processes.[11] The previous studies have undoubtedly focused on radial cell migration. In contrast, cell movement in our system is more similar to tangential cell migration than to radial migration for two reasons. First, we used granule cells from two-day-old rat cerebella; second, there were no glial cell processes outside the reaggregate cell cultures. If our reasoning is correct, NT-3 might inhibit tangential cell migration, but might either not affect or might promote migration along the radial glial processes. In fact, NT-3 might be one of the molecules responsible for the change from tangential to radial migration.

Another possible explanation for the differences in results is that cells might respond differently to NT-3 in our culture system than in previous studies. The direction in which growth cones turn in response to neurotrophins can be changed by altering cyclic nucleotide second messenger levels.[34,35] Since the environment in our culture system is different from that in previous studies, the effect of NT-3 might be different.

Another possibility could be that an increased maturation of granule cells or a transition from a tangential to a radial type of migratory pattern might have resulted in a decreased ability of granule cells to move, since astrocytes are not present in our preparation. Further work is required to address these issues.

In our studies, the inhibitory effects of NT-3 on the speed of cell soma movement and the migration index were evident after 24 and 36 h, respectively. This time-course is similar to the effects of BDNF on the neurite extension. The mechanisms by which NT-3 inhibits cell movement are unknown.

Since cell migration is thought to be regulated by the activity of cytoskeletal, membrane and adhesion systems,[24] NT-3 might affect one of these systems.

Differential effects of brain-derived neurotrophic factor and neurotrophin-3 on granule cells

Although neurotrophins are generally thought to have identical biological effects on cells, we have shown, in this study, that BDNF and NT-3 have different effects on granule cells. BDNF affected the motility of the growth cone, and in contrast, NT-3 affected the motility of cerebellar granule cell soma. It is possible that BDNF and NT-3 were acting on different populations or types of granule cells, since cells with extending neurites and moving cells might be different. This possibility is rather unlikely, since the differential effect of neurotrophins on the motility of the cell soma and growth cone of an identified neuron was similar to the overall differential effect of neurotrophins on granule cells. Segal *et al.*[31] have shown that BDNF, but not NT-3, promotes granule cell neurite extension, and that NT-3, but not BDNF, enhances granule cell neurite fasciculation or branching on poly-ornithine-coated coverslips in reaggregation cell cultures. Furthermore, McAllister *et al.*[22] showed that BDNF and NT-3 have opposite effects on cortical dendritic growth. Song *et al.*[35] also showed that BDNF and NT-3 have opposing effects on growth cone guidance.

How do BDNF and NT-3 exert their different effects on granule cells? One possibility is that the two neurotrophins use different signaling pathways. It is known that BDNF acts through cyclic-AMP-dependent pathways, while NT-3 acts through cyclic-GMP-dependent pathways.[34] A less likely possibility is that TrkB and TrkC are distributed differently on growth cones and cell somata. However, we have demonstrated by immunocytochemistry that both TrkB and TrkC are present on granule cell growth cones and cell somata in the reaggregation cell cultures (data not shown).

CONCLUSION

In summary, we analysed the effects of exogenous neurotrophins on cell movement and neurite extension, using a glial-free reaggregation culture prepared from two-day-old rat cerebellar granule cells. This culture enabled us to analyse the direct effects of exogenous neurotrophins on granule cells without the influence of glial cells. We showed that NT-3, but not BDNF, inhibited the motility of cell somata, while BDNF, but not NT-3, promoted the motility of the growth cones. This is the first direct evidence that NT-3 inhibits granule cell movement.

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