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Research report

Regulation of pontine neurite morphology by target-derived signals

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Abstract

The molecular cues that regulate neurite morphology within the target environment are key to the formation of complex neural circuitry. During development of the ponto-cerebellar projection, pontine fibers sprout and form elaborate arbors within the inner cerebellar layer prior to arrival of their target cells, the cerebellar granule neurons. Here, we describe the biochemical fractionation of two granule neuron-derived factors that stimulate elaboration of pontine neurites. These factors were identified using a dissociated pontine bioassay and biochemically fractionated from granule cell (GC) conditioned medium (GCCM). One of the factors, STIM1, is a protein with a molecular weight greater than 30 kDa that is distinct from known neurotrophins. The other, STIM2, is a small, protease-resistant molecule with an estimated molecular weight below 1 kDa. We show that these factors stimulate pontine neurite elongation both independently and cooperatively and thus may contribute to the formation of elaborate pontine arbors within the cerebellar cortex.

Theme: Development and Regeneration
Topic: Process outgrowth, growth cones, and sprouting

Keywords: Neurite outgrowth; Development; Target-derived factors; Basilar pontine nuclei; Granule cells; Cerebellum

1. Introduction

The development of the central nervous system relies on multiple, complex signals between growing neurons and the surrounding cells. These signals guide extending axons toward their targets [36], support arborization in the target region (see, for example, Refs. [6,37]) and provide cues for synapse formation [39]. The murine cerebellar system provides an excellent setting to investigate the molecular biology of these signals, as the cerebellum contains relatively few cell types, and these have been well characterized at the developmental and anatomical level (reviewed in Ref. [13]).

There are two main afferent populations that enter the cerebellum, pontine mossy fibers, and olivary climbing fibers [22,26]. While pontine mossy fibers target the abundant cerebellar granule neurons, the olivary fibers synapse on Purkinje cells. The pontine afferents that are the focus of this study arrive at the cerebellum around the time of birth. Here, pontine afferents defasciculate and begin to form branches and various filopodia-like structures that—with time—may even reach into neighboring folia [2,17,21,26]. Concurrently, granule cell (GC) precursors undergo multiple divisions in the outer germinal layer and the postmitotic cells migrate in to populate the emerging granule layer, which the first cells reach around postnatal day 3 (P3) (for illustration, see Fig. 7) [13]. Thus, pontine afferents enter the target area and begin to form arbors prior to making direct contact with their target cells. From the time of birth and over the course of the next 2–3 weeks, pontine afferents continue to develop elaborate arbors and form synaptic contacts with granule neurons through large swellings that develop into glomeruli [21,22].

A number of signals have recently been shown to play a role in the development of synaptic connections between afferents to the cerebellum and their targets. For instance, Semaphorin 3A expressed by Purkinje cells has an inhibitory effect on the growth of pontine mossy fibers but not olivary climbing fibers [28,35]. At the same time, BDNF expressed by granule cells has a trophic effect on the outgrowth of pontine mossy fibers [27]. Other granule cell factors such as

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Neuregulin, Wnt-7a, and Neuroligin [11,25,32] play a role in stimulating synapse formation between pontine fibers and granule cells.

During our experiments with pontine explants, we have observed that cerebellar conditioned medium (CCM) promotes pontine neurite outgrowth, a phenomenon also noted by Zhang and Mason [38]. In order to further investigate the role of such target-derived activities in promoting outgrowth of pontine afferent fibers, we developed an in vitro bioassay system using dissociated pontine neurons. This bioassay was designed to detect outgrowth-promoting activities in a short-term, low-density culture system where a majority of outgrowth is non-fasciculated.

Using this bioassay, our analyses revealed that cerebellar cells, particularly granule neurons, release activities that rapidly modulate pontine neurite outgrowth and morphology in vitro. We have partially purified two such activities through biochemical fractionation and found that known neurotrophins and other candidate molecules do not appear to substitute for their effect. We propose that these activities may constitute target-derived signals that drive the formation of elaborate pontine arbors and thus contribute to pontine afferent development prior to synapse formation with granule cells.

2. Materials and methods

2.1. Dissociated pontine neurons

Dissection of pontine explants was performed as described previously [1], and the dissociation protocol was based on previously described methods for granule cell isolation [1,12]. Pontine nuclei from three to five P0 or P1 CD-1 mice were collected and sliced into four to five pieces each. Tissue pieces were incubated for 10 min at 37 °C with 1 mg/ml trypsin (Sigma, St. Louis, MO) and 0.1 mg/ml DNase I (Roche, Indianapolis, IN), followed by the addition of DNase I (to 0.3 mg/ml) and soybean trypsin inhibitor (SBTI, to 0.7 mg/ml, Sigma) for a final 5-min incubation. Cells were triturated between and after incubations, using a fire-polished Pasteur pipet. The cell suspension was diluted with HEPES-buffered Hank's Balanced Salt Solution (HHBSS, 10 mM HEPES pH 7.2, Invitrogen, Carlsbad, CA), spun through a cushion of 4% BSA (ICN Biomedicals, Aurora, OH) at 400 × *g*, 4 °C for 10 min, and the pelleted cells were resuspended in serum-free medium (SFM). SFM consisted of Neurobasal medium with 2 mM GlutaMAX-1, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 × B-27 Serum-Free Supplement (all from Invitrogen), and 10 ng/ml recombinant human BDNF (Peprotech, Rocky Hill, NJ). Viable cells were counted by Trypan blue exclusion and plated at 5000 cells/cm^2 (300 μl volume) in prepared Lab-Tek Permanox eight-well Chamber Slides that had been coated with 1.5 μg/ml poly-L-ornithine (Sigma) for 30 min and 25 μg/ml laminin (Roche) for 2 h. Cultures were incubated at 37 °C, 85% relative humidity, and 5% CO_2.

2.2. Pontine bioassay

Basilar pontine nuclei (or granule neurons) were dissociated and plated as above. Proportions of conditioned media and fractions being tested ranged from 0.6% to 20% of the bioassay volume. After incubation for 18–24 h, cells were stained with CellTracker green CMFDA (Molecular Probes, Eugene, OR) for 30 min at 37 °C, rinsed in Neurobasal, and fixed for 30 min in 4% paraformaldehyde/4% sucrose/1 × Dulbecco's phosphate-buffered saline (PBS, Invitrogen). Cells were washed using PBS and the slides were mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

To evaluate activity during the biochemical fractionation, neurite outgrowth was scored semi-quantitatively on a scale from 0 (control) to +3 (highly stimulatory). Fractions subjected to further study were additionally quantified using Openlab v.2 software from Improvision (Lexington, MA). For this purpose, bioassay images were acquired using a Nikon PCM2000 confocal laser system, with 8–12 non-overlapping images collected for each bioassay replicate. The number of images per bioassay varied between experiments, but not within a given set of experimental replicates. On each image, the number of cells was counted and a line tracing tool was used to deduce the total length of all neurites (excluding short processes less than 30 μm). For each image, total neurite length divided by the number of cells equaled the total neurite length per cell. The average and standard deviation were calculated from three or more replicates of each experiment.

2.3. Preparation of conditioned medium

Biochemical fractionation of conditioned medium was simplified by decreasing the amount of bovine serum albumin (BSA) found in the B-27 supplement of SFM. To achieve this, a 2 × stock of B-27 in Neurobasal was passed over a Blue-Sepharose column (Amersham Pharmacia, San Francisco, CA), applying 2 ml of B-27 for every 1 ml of sepharose. The column was pre-equilibrated with HMGlu (10 mM HEPES pH 7.2, 1 mM $MgCl_2$, 25 mM glucose, 100 mM NaCl)—a solution that simulates the buffering capacity and ionic strength of Neurobasal—and one column volume (CV) of Neurobasal. The flow-through was diluted 1:20 in Neurobasal (to 0.1 × B-27) with 2 ng/ml BDNF, 20 μM AEBSF (Calbiochem, San Diego, CA), GlutaMAX, penicillin, and streptomycin to make conditioning medium (CM). The differences between CM and SFM had no effect on the growth of the high density cultures used for conditioning (data not shown). CM was also used as a negative control in bioassays.

To make CCM, whole cerebella were dissected from P5–P10 CD-1 mice and cut into four to five pieces sagitally.

Approximately 60 mg tissue was placed on a tissue culture insert with a 0.4-μm filter membrane and incubated in 3.5 ml of CM. Cultures were incubated for up to 15 days, with medium outside the tissue culture insert (~ 80%) being harvested and replaced every 2–3 days. Harvested media was passed through a 0.2-μm filter, supplemented with AEBSF to 100 μM, flash frozen and stored at − 80 °C.

To make granule cell conditioned medium (GCCM), whole cerebella were dissociated and plated as described below. Every 2–3 days, 80% of the conditioned medium was harvested and each plate refilled to its starting volume with fresh CM. Harvested medium was treated as for CCM. "Large cell" conditioned medium was made the same way, using the "large cell" population collected from the dissociation below.

2.4. Dissociated granule neurons

The protocol for dissociating GCs was based on previously published methods [1,12]. Whole cerebella were dissected and dissociated as described for the pontine dissociation. After trituration, GCs were mixed 1:1 with a solution of 40% Percoll (Amersham Pharmacia)/0.2% glucose/1 × PBS, overlaid onto an equivalent volume of 40% Percoll/glucose/PBS, and spun at 1400 × *g* for 10 min at 4 °C. The pelleted cells were diluted in HHBSS, spun through a BSA cushion, and finally resuspended in CM. Cells were plated at 1–2 million cells/cm^2 on tissue culture plates pretreated with poly-L-ornithine and laminin. When GCs were used for bioassays, cells were resuspended in SFM instead of CM and plated at 5000 cells/cm^2 in prepared Lab-Teks.

When comparing "large cell" and granule cell populations using this protocol, the "large cell" fraction was harvested from the interface of the 20% and 40% Percoll layer after the first spin. These cells were diluted with HHBSS and passed through a BSA cushion before plating in CM. To further purify the GC fraction, the GC pellet was diluted and passed through another Percoll gradient, followed by a BSA cushion spin, and plated in CM.

2.5. Immunofluorescence

After fixation and washing, cells were held for 1 h in PBS with 10% heat-inactivated goat serum (Invitrogen) and 0.1% Triton X-100 (GS/PBS/Triton), followed by overnight incubation with GS/PBS/Triton containing 0.5 μg/ml polyclonal anti-synapsin I (Chemicon, Temecula, CA), 0.5 μg/ml monoclonal anti-microtubule associated protein 2 (MAP-2) (Roche), and/or 75 μg/ml polyclonal anti-GFAP (Sigma). Cells were washed, incubated for 2 h in GS/PBS/Triton with 7.5 μg/ml Cy2-conjugated goat anti-rabbit IgG for the polyclonal or 3.75 μg/ml Cy3-conjugated goat anti-mouse IgG for the monoclonal (both from Jackson Laboratory, Bar Harbor, ME), washed again, and mounted as usual.

2.6. Cell viability

The manufacturer's protocol for a viability assay (Live/Dead assay, Molecular Probes) was used to assess survival in the pontine bioassay. The green fluorescent dye supplied with the kit was replaced by green CMFDA to allow for scoring of neurite outgrowth. Pontine bioassays with and without 20% GCCM were tested in triplicate. For each well, 10 non-overlapping fields of view were scored for outgrowth and the number of red (dead) versus green (live) cells was counted and used to calculate the ratio of live cells to total number of cells. Percent survival per well was calculated from an average of ten fields, and three to six wells were tested for each condition.

2.7. Candidate molecules

Candidate molecules were resuspended according to the manufacturer's instructions and tested in the ranges listed in Table 1. All reagents were from Peprotech, with the exception of Neuregulin (Heregulin β1, Neomarkers, Fremont, CA), carboxy-terminal Slit2 (c-Slit2) [3], and WNT-7a [11].

For WNT-7a testing, HEK293 cells were grown in a-MEM with 10% FBS, 1 mM Glutamine, 1 × non-essential amino acids, and penicillin and streptomycin as above (all Invitrogen), and transfected using Lipofectamine Plus (Roche). Wnt-7a was expressed by transiently transfected HEK-293 cells plated on pretreated Lab-Teks. At 24–48 h after transfection, the medium was replaced with SFM and dissociated pontine neurons were added as for the pontine bioassay. Cells were labeled using anti-synapsin antibody and anti-hemagglutinin (HA, Roche) antibody recognizing an HA-epitope tag present on WNT-7a. Control cultures were transfected with a Green Fluorescent Protein expressing vector, pCS2.

Table 1
Candidate molecules tested for stimulation of pontine neurite growth

β-NGF (1–100 ng/ml)	VEGF (1–100 ng/ml)
CNTF (0.1–10 ng/ml)	b-FGF (0.1–10nM)
BDNF (1–100 ng/ml)	a-FGF (0.1–10nM)
GDNF (1–100 ng/ml)	FGF-9 (0.1–10nM)
NT4 (1–100 ng/ml)	
NT3 (1–100 ng/ml)	
BDNF + β-NGF	Neuregulin (0.1–10 nM)
BDNF + CNTF	c-Slit2 (0.5–50 mM)
BDNF + GDNF	WNT-7a
BDNF + NT4	
BDNF + NT3	

Neurotrophins β-NGF, CNTF, BDNF, GDNF, NT4, and NT3 were tested in the presence or absence of BDNF under standard conditions as described in the Materials and Methods. Other factors were tested in the presence of BDNF. c-Slit2 is the C-terminal 60 kDa fragment of human Slit2. Neuregulin is the 30 kDa extracellular domain of human Heregulin β1. WNT-7a was expressed by transient transfection of HEK-293 cells that were co-cultured with pontine neurons under standard conditions. None of the factors listed in this table were able to substitute for CCM or GCCM in stimulating pontine neurite growth and filopodia formation.

2.8. Initial size fractionation of conditioned medium

As a first step, CCM or GCCM was separated into two fractions using an Amicon ultrafiltration cell with a 30 kDa cutoff membrane (Millipore, Bedford, MA). The 30 kDa retentate includes the large molecular weight component(s) described in the text as STIM1. The 30 kDa filtrate was then applied to a 1 kDa cutoff membrane (Millipore). The 1 kDa filtrate contains the stimulatory component(s) described in the text as STIM2. To generate appropriate control media for bioassays, CM was also subjected to fractionation into 30 kDa retentate, 30 kDa filtrate, 1 kDa retentate, and 1 kDa filtrate fractions. Any samples that were not used immediately were flash frozen and stored at − 80 °C.

2.9. Fractionation of STIM1

The concentrated 30 kDa retentate was dialyzed overnight against 1.5 M ammonium sulfate in HMGlu at 4 °C. After dialysis, the medium was centrifuged, sterile filtered, and applied to a 1.66-ml POROS PE (Applied Biosystems, Foster City, CA) phenyl ether column run at 1 ml/min. Starting buffer was the same as dialysis buffer, and the column was eluted with a 12-CV gradient from 1.5 to 0 M ammonium sulfate in HMGlu. Fractions of 0.75 ml were collected, dialyzed against HMGlu overnight, and bioassayed.

Peak fractions were concentrated to 0.5 ml using a 30 kDa cutoff membrane and applied to a Superdex 200 HR gel filtration column (S200, 10 × 300 mm, Amersham Pharmacia). The column was run at 0.3 ml/min in HMGlu, and 0.5-ml fractions were collected. Fractions were bioassayed directly or flash frozen for storage at − 80 °C. A set of gel filtration standards (BioRad, Hercules, CA) was run under the same conditions to determine the elution time for known proteins.

2.10. BDNF immunoblotting

Presence of BDNF in fractionated GCCM was tested by immunoblotting with a polyclonal antibody to BDNF (Research Diagnostics, Flanders, NJ). Recombinant BDNF (Peprotech, as above) served as a positive control and was detectable to 10 ng. GCCM fractions tested included: 2 μl of a 54 × concentrated 30 kDa retentate of GCCM fractionated by S200 gel filtration, 10 μl of peak fraction 28 from the same S200 column, and 4 μl each of the following samples (all concentrated 20 fold): GCCM 30 kDa retentate, GCCM 1 kDa retentate, control medium 30 kDa retentate, and control 1 kDa retentate.

2.11. Fractionation of STIM2

A BSA column was made by coupling fatty acid free BSA (ICN Biomedicals) to a carbonyldiimidazole activated support (Reacti-Gel TSK HW-65, Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions, with a final ratio of approximately 4 mg BSA to 1 mg beads. After equilibration of the column with HMGlu and rinsing with Neurobasal, 10 ml of the 1 kDa filtrate described above was applied per 1 ml beads and the flow-through collected into a single fraction (1 kDa BSA-FT). As a control for the bioassay, the 1 kDa filtrate of CM was also passed over a BSA column to produce a control 1 kDa BSA-FT.

2.12. Enzymatic digestion of STIM2

Stimulatory and control 1 kDa BSA-FT samples were digested with 100 μM chymotrypsin (CT, Roche) for 1 h at 37 °C and the digestion stopped with 100 μM SBTI. To remove the CT and SBTI, at 25 and 20.1 kDa, respectively, samples were centrifuged on individual spin columns with a 3 kDa molecular weight cutoff membrane (Millipore) at 6500 × *g*, 4 °C for 2 h, after which the filtrate were tested by bioassay. To confirm the enzymatic activity of CT under these conditions, 0.7 μM BSA was added to stimulatory 1 kDa BSA-FT and treated as above. After incubation, digestion was stopped by adding SDS sample buffer and boiling at 100 °C. Samples were analyzed on a 10% SDS–PAGE gel. Proteinase K treatment of stimulatory and control 1 kDa BSA-FT was performed as described above, using 1 μM Proteinase K (Roche) and incubating at 37 °C for 1 h. Activity and digestion of BSA were analyzed as for chymotrypsin.

2.13. Heat treatment of stimulatory activities

To test for heat stability or sensitivity of STIM1 and STIM2 in the S200 peak fraction and the 1 kDa BSA-FT, samples were incubated at either 75 °C or 95 °C for 15 min and chilled on ice. STIM1 heat-treated samples were centrifuged to remove precipitates, and the supernatants were assayed. Heat-treated samples of STIM2 and control 1 kDa filtrate were loaded onto 3 kDa cutoff spin columns to remove any precipitates and the filtrates were tested by bioassay.

3. Results

To investigate mechanisms of retrograde signaling during target innervation, we have developed an in vitro model system using two natural synaptic partners, the pontine neuron from the basilar pontine nucleus (BPN) and its target cell, the cerebellar granule neuron. In our in vitro culture system, conditioned medium was prepared by culturing postnatal days 6–8 (P6–P8) mouse cerebellum in defined medium. Dissociated pontine neurons were prepared from the BPN isolated from newborn mice and plated at low density (5000 cells/cm^2) on a highly permissive growth substrate. To assess the role of conditioned

medium, these pontine neurons were cultured for 20–24 h in defined medium in the presence of BDNF as the only neurotrophin and supplemented with either control medium or CCM. Pontine neurons grown in the presence of 6% CCM extended neurites that were longer, thicker, and more branched than cultures with BDNF alone (Fig. 1A–D). Quantitation of neurite length revealed a four- to fivefold increase in total neurite length per cell in the presence of CCM (Fig. 1G). We refer to these neurites as presumptive axons because they stained positive for the synaptic vesicle protein synapsin I and the neuronal marker MAP-2, particularly within the cell soma and proximal processes. In addition, pontine neurons that exhibited a response to CCM had a morphology characteristic of projection neurons with one to three short processes (presumably dendrites) and one long process (the presumptive axon). In addition to the morphological changes noted above, we also observed that CCM induced an increase in the number of branchlets or spine-like structures along the presumptive axon shaft (Fig. 1E–F). These results together show that target-conditioned medium has a rapid stimulatory effect on pontine neurite morphology.

Originally, we had observed this stimulatory activity with pontine explants that were cultured either in the presence of granule cells or CCM. However, pontine explants exhibited varying degrees of fasciculation, which complicated quantitation of individual neurite growth. Thus, we chose to use dissociated cultures plated at a low density to eliminate fasciculated growth and facilitate quantitation.

3.1. Stimulatory activity is produced by granule neurons

Around the time of birth, pontine afferents reach the cerebellum, defasciculate, and begin to form extensive axonal arbors. This process continues during the first two postnatal weeks as the cerebellar cortex expands. Consistent with this period of pontine afferent growth, we found that cerebella isolated from P0 through P14 mouse pups produced, although at declining levels, an activity that stimu-

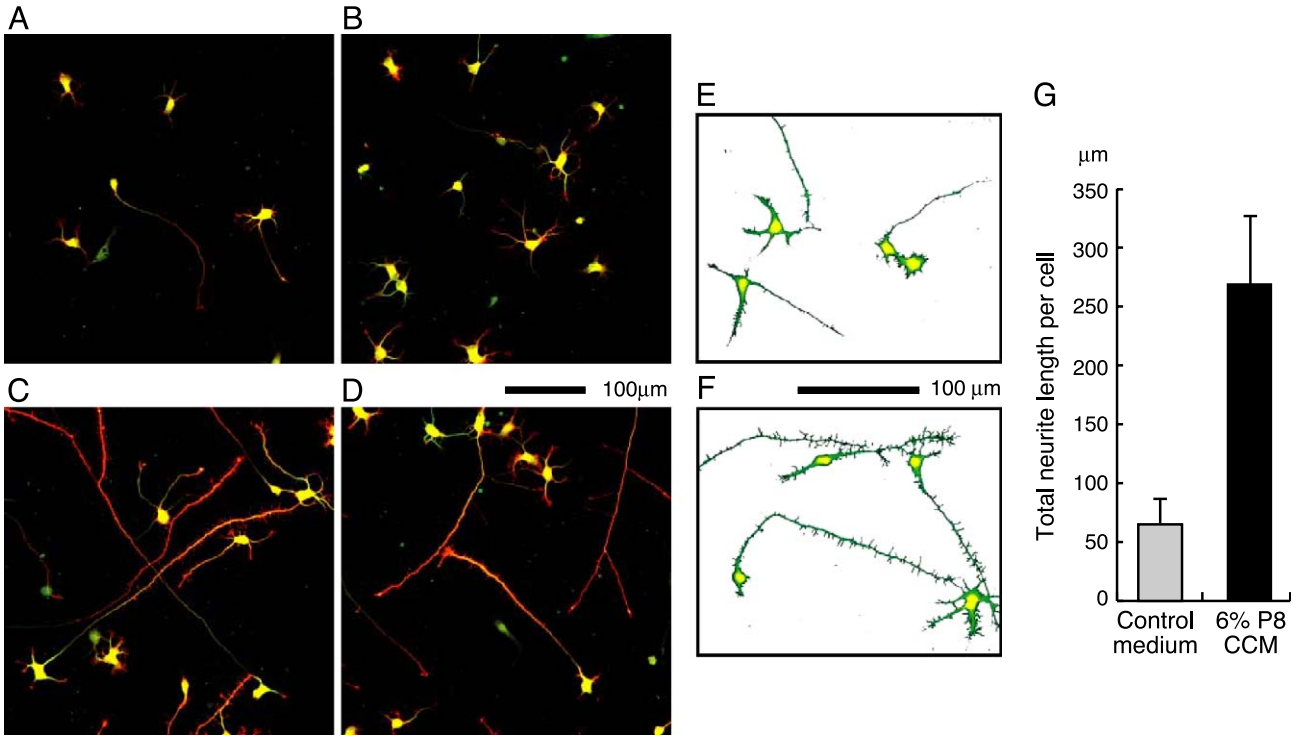

Fig. 1. Stimulation of pontine neurite growth and filopodia formation by cerebellar conditioned medium. Dissociated pontine neurons were grown in defined medium in the absence (A, B) or presence (C, D) of 6% cerebellar conditioned medium (CCM) prepared from P8 mouse cerebellum (see Materials and Methods for details). Pontine neurons were fixed after 20 h in culture and stained with anti-MAP2 antibody (green) and anti-synapsin I antibody (red). Images were acquired using confocal microscopy. (E, F) Filopodia formation is more clearly visualized at higher magnification and strongly enhanced in the presence of 6% CCM (F) relative to control medium (E). The cells shown in E and F were selected because of their similar length, and thus are not representative of the length effect of CCM. These cells demonstrate that the enhanced filopodia formation is not simply due to increased neurite length but is visible even on short processes in CCM-treated cultures. Cells were stained with the cell-permeable fluorescent dye green CMFDA prior to fixation. (G) Quantitation of pontine neurite length. Neurites longer than 30 μM were traced using computer-assisted morphometric analysis and the total neurite length divided by the number of cells was calculated for each field. For each condition, an average of total neurite length per cell was calculated from 10 such fields (see Materials and Methods for further details). The data show the average of three experiments and the error bars represent the S.E.M. Statistical significance was determined using Student's t test (unpaired): $p < 0.005$.

lated pontine neurite growth as described above (data not shown). This implies that the cells that secrete this activity must be present at early as well as at later stages of cerebellar development.

The developing cerebellum contains several neuronal and glial cell types. To determine what cells were producing this activity, dissociated cerebella were separated into two fractions by Percoll density gradient centrifugation. This procedure yielded a ''large cell fraction'' enriched in glia (19% glial fibrillary acid protein [GFAP] positive cells) and a granule cell fraction with few glial contaminants (1% GFAP positive cells). These two fractions were plated separately and used to prepare conditioned media that were then tested for their ability to regulate pontine neurite outgrowth. The ''large cell'' or glia conditioned medium had a modest stimulatory effect on pontine neurite growth, relative to the GCCM that stimulated neurite growth up to fivefold in a dose-dependent manner (Fig. 2A). This result suggests that granule neurons may indeed be the primary source of this activity. Furthermore, the presence of this activity from P0 to P14 indicates that it is produced by granule cells in the external germinal layer and the nascent internal granule cell layer.

Control experiments showed that GCCM had no significant effect on plating efficiency or cell density (Fig. 2B). A vital cell stain was used to determine the effect of GCCM on pontine neuron survival and revealed a small increase (Fig. 2C). However, this effect on survival is not sufficient to explain the increase in neurite length as the

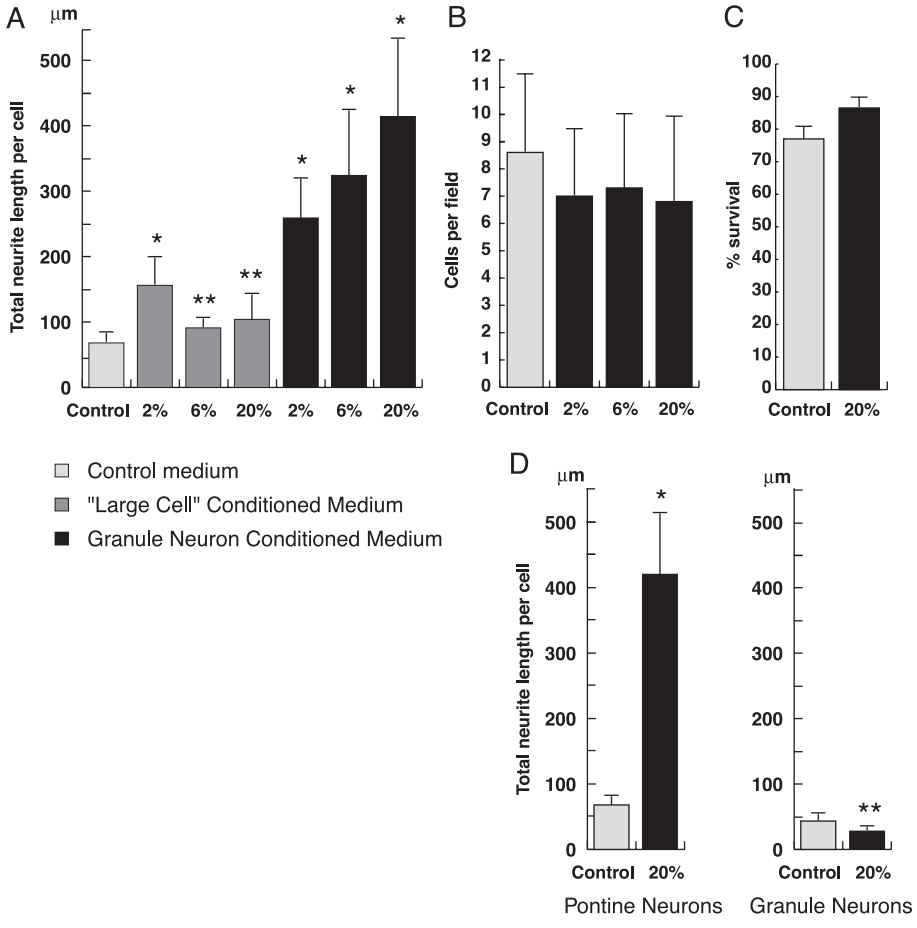

Fig. 2. Granule cells produce an activity that stimulates pontine neurite extension. Cerebellar cells were fractionated into two populations, the ''large cell'' fraction rich in glial cells (19% glial fibrillary acidic protein (GFAP) positive cells) and the granule cell fraction (1% GFAP positive cells and >95% MAP-2 positive cells). (A) Pontine neurons were grown in absence (control) or presence of increasing amounts (2–20%) of conditioned medium derived from these cell populations. Pontine neurons were stained with green CMFDA, fixed and quantified as described in Fig. 1 and under the Materials and Methods. Statistical significance (Student's *t* test, unpaired): $*p < 0.05$ for column 1 versus columns 2, 5, 6, or 7. No statistical significance ($**p > 0.05$) for column 1 versus 3 or 4. (B) The number of cells per field was determined from the experiments shown in (A). No statistical significance for control versus column 2, 3, or 4. (C) Using the same growth conditions as in (A), the survival of pontine neurons was determined in absence (control) or presence (20%) of 20% granule cell conditioned medium (GCCM). The fraction of surviving cells was visualized using a live/dead cell assay as described in the Materials and Methods ($p < 0.05$). (D) GCCM stimulates the growth of long neurites from pontine cells but not granule cells. Pontine and granule neurons were grown under the same conditions in absence (control) or presence (20%) of GCCM for 20 h. Cells were stained and analyzed as described above. Statistical significance: $*p < 0.005$ versus control. No statistical significance $**p > 0.05$ versus control. The histograms show the average of three to four experiments and the error bars represent the S.E.M.

fraction of cells with quantifiable neurites typically constitutes 50–70% of the cell population (data not shown), considerably more than the approximately 10% increase in survival.

To gain insight into whether the stimulatory activity could promote extension of long neurites in granule cells themselves, we tested granule cells in the assay system used for pontine neurons. Under these conditions, the number of granule cells that were plated was several fold below the number of cells otherwise necessary to produce a response in pontine neurons. In untreated cultures, granule and pontine neurons produced neurites of similar average length (Fig. 2D). However, in the presence of GCCM pontine neurite length increased by more than fivefold and, on average, pontine neurites were 10 times longer than granule neurites (Fig. 2D). Although localized autocrine effects on granule cells grown at low density cannot be excluded, this experiment clearly shows that extension of long neurites (>400 μM) in response to GCCM is a hallmark of pontine neurites that is not seen with granule cells (<40 μM).

Together, these experiments show that granule neurons secrete an activity during early postnatal development that stimulates the elaboration of pontine neurites. This stimulation has been observed with both dissociated cells and pontine explants and thus may constitute a paracrine signal between granule neurons and ingrowing pontine afferents.

3.2. Roles of neurotrophins and other candidate molecules in regulating pontine axon growth

Given the interesting properties of this paracrine signal, we decided to characterize it in more detail. First, we used our in vitro assay to examine a panel of candidate molecules that had previously been shown to display similar activity. Neurotrophins have been reported to play an important role as autocrine or paracrine trophic signals during target innervation [4,6,15] and to influence the shaping of dendritic and axonal arbors of cerebellar cells [23,33,34]. The neurotrophins BDNF, NGF, NT3, and NT4/5 are present at various stages during cerebellar development [19] and, in particular, BDNF is expressed by granule cells and recently has been reported to stimulate pontine neurite growth [27]. Our standard assay contained 10 ng/ml BDNF and increasing the concentration of BDNF further did not produce enhanced outgrowth (data not shown). By contrast, addition of GCCM (in presence of BDNF) resulted in a dose-dependent stimulation of outgrowth, suggesting that the stimulatory activity in GCCM was not BDNF. We also tested the neurotrophins β-NGF, NT3, NT4, GDNF, and CNTF in the absence or presence of BDNF, but none of these appeared to recapitulate the stimulatory effect of GCCM. Wnt7a has been reported to induce axonal spreading and growth cone enlargement in granule neuron and pontine explant cultures [11]. These studies did not report effects on neurite extension, consistent with our observations that co-culture of Wnt7a expressing cells with pontine neurons did not enhance pontine neurite extension. Likewise, other candidate molecules reported to stimulate neurite extension or branching in various systems [3,14,30] could not substitute for GCCM in stimulating pontine neurite extension (Table 1). In light of these results, we speculated that the activity might be novel and decided to proceed with a biochemical fractionation to gain further insight into the nature of the stimulatory activity.

3.3. Biochemical fractionation of GCCM

For biochemical fractionation studies, we generated large quantities of conditioned medium from cerebellar granule cells, as GCCM exhibited lower protein complexity than CCM by protein gel analysis (data not shown). In addition, conditioning medium was passed through a Blue-Sepharose column to remove the majority of BSA, which constituted more than 80% of total protein in B-27 conditioned medium (Fig. 3A) and hampered subsequent fractionation steps.

The first step in the fractionation of GCCM was to separate the activity into two components using an ultrafiltration membrane with a 30 kDa cutoff (Fig. 3B). The concentrated retentate (>30 kDa) was dialyzed against 1.5 M ammonium sulfate and centrifuged to remove residual

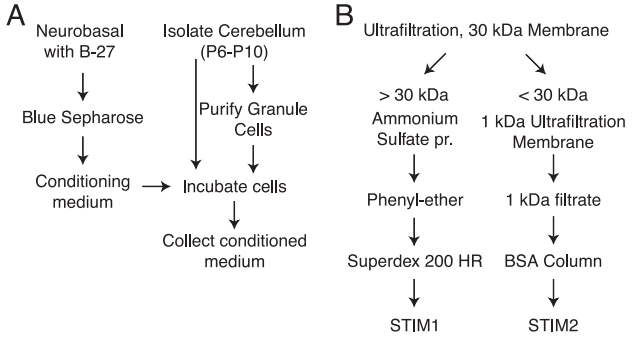

Fig. 3. Overview of fractionation procedure. (A) Neurobasal medium containing 2× B-27 growth supplement was partially depleted of BSA on a Blue-Sepharose column and diluted to 0.1× B-27 in Neurobasal for preparation of conditioning medium. Cerebella (for CCM) and granule cells (for GCCM) were prepared as described in the Materials and Methods and medium was harvested every 2–3 days. (B) Conditioned medium was split into two fractions using an ultrafiltration membrane with a 30 kDa cutoff. The 30 kDa retentate was dialyzed against 1.5 M ammonium sulfate and fractionated by phenyl-ether chromatography. Fractions containing peak activity were concentrated and further fractionated by Superdex 200 HR chromatography yielding a partially purified fraction containing STIM1 activity. The activity in the <30 kDa fraction was passed through an ultrafiltration membrane with a 1 kDa cutoff. The resulting 1 kDa filtrate was passed over a column containing delipidated BSA and activity was recovered in the flow-through.

macromolecular material. The activity was then fractionated by phenyl-ether and size-exclusion chromatography. The sizing column yielded two peaks of activity at 130 and 55 kDa, respectively (Fig. 4A). The 130 kDa peak only produced a modest stimulation and potentially could correspond to the extracellular domain of L1, which has previously been shown to be produced by cultured granule cells [18]. The more abundant 55 kDa activity, which is referred to as STIMulatory activity 1 or STIM1, produced a four- to fivefold increase in pontine neurite length that was comparable to the maximum stimulation obtained with crude GCCM (Fig. 4B). Furthermore, STIM1 activity was inactivated by heating to 75 °C for 15 min, supporting the hypothesis that STIM1 is likely to be a protein.

Since BDNF was the only neurotrophin that was included in our standard assay, we tested whether STIM1 could promote neurite extension independently of exogenous BDNF. STIM1's effect on neurite outgrowth was not significantly changed by omission of exogenous BDNF, whereas neurite length in the control reaction was reduced by 35% upon removal of BDNF (Fig. 4C). By Western blot analysis, we could not detect BDNF in the 55 kDa fraction or in the 30 kDa retentate (data not shown). These observations suggest that STIM1 can act independent of the neurotrophin BDNF to modulate pontine axon morphology.

The filtrate (or flow-through) from the ultrafiltration (30 kDa cutoff) was also found to be active. Additionally, this activity passed through a membrane with a 1 kDa cutoff, suggesting a size for the active component around or less than 1 kDa (Fig. 3B). We refer to this activity as STIMulatory activity 2 or STIM2. Analysis of STIM2 activity revealed a maximum stimulation of pontine neurite extension of about four- to fivefold, which is comparable to the maximum stimulation obtained with STIM1 or crude GCCM. Interestingly, STIM2 activity was not eliminated by heating to 75 °C and even showed activity after heating to 95 °C (Fig. 5A). Thus, STIM1 and STIM2 can be distinguished by at least two criteria—size and heat stability—suggesting that they are distinct molecules.

We tested STIM2 for binding to delipidated BSA, since many small lipophilic molecules can bind to BSA and to determine whether STIM1 in the 55 kDa fraction could be due to a small molecule such as STIM2 binding to BSA and being carried along in the fractionation. However, STIM2 showed no significant binding to BSA, thus excluding the possibility that STIM1 is caused by STIM2 binding to BSA.

To determine whether STIM2 could be a small peptide, STIM2 was subjected to proteolytic digestion. Chymotrypsin treatment appeared to have no major effect on the ability of STIM2 to promote neurite growth (Fig. 5B). As a control for the efficiency of the proteolytic digestion, BSA added to a STIM2 fraction was found to be extensively degraded (Fig. 5B). In addition, experiments using proteinase K, a protease that is less site-specific than chymotrypsin, gave comparable results (data not shown). These experiments provide evidence that STIM2 is not likely to be a polypeptide.

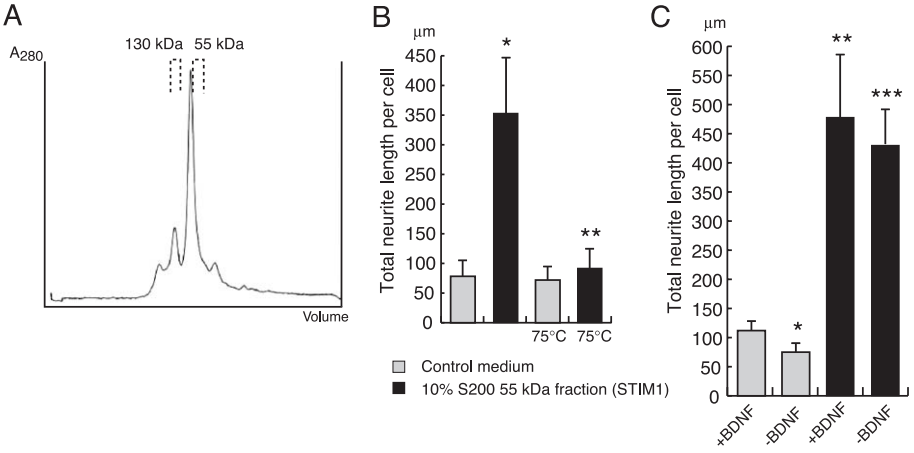

Fig. 4. Analyses of STIM1 activity from Superdex S200 fraction. (A) Chromatogram of Superdex 200 HR (S200) fractionation. Two peaks of activity were detected eluting at positions corresponding to molecular weights of 130 and 55 kDa, respectively, according to size markers fractionated under similar conditions. The 55 kDa activity eluted in the tail of the large BSA peak. Activity was scored semi-quantitatively on a scale from 0 to +++ (0 indicated no activity above background, +++ was positive control GCCM). The 55 kDa fractions (indicated with a bracket) showed activity comparable to the input fraction, ++ to +++. The 130 kDa fractions (indicated with a bracket) were less active than the input fraction, + to ++. (B) Heat inactivation of STIM1 activity in the 55 kDa S200 fraction. Fraction and control medium were heated to 75 °C for 15 min and centrifuged to remove debris. Statistical significance (Student's *t* test, unpaired): $*p < 0.005$ for column 1 versus 2. No significance for heat-treated samples: $**p > 0.05$ for column 3 versus 4. (C) To test for the dependence on BDNF, pontine neurite growth was tested in presence or absence of exogenous BDNF. The STIM1 fraction was also assayed in presence or absence of exogenous BDNF. Western blot analysis revealed that the 55 kDa STIM1 fraction does not contain BDNF produced by granule cells and potentially carried along with STIM1 during the fractionation (data not shown; see Materials and Methods for details). Statistical significance: $*p < 0.05$ for column 1 versus column 2, $**p < 0.005$ for column 1 versus column 3. No statistical significance between column 3 and 4: $***p > 0.05$. The data show the average of four experiments and the error bars represent the S.E.M.

Adenosine, guanosine, and their triphosphate derivatives ATP and GTP are among the few small molecules that have been reported to enhance neurite outgrowth [9,10,29]. However, experiments with ATP and GTP did not reveal any outgrowth promoting activity with pontine neurons (data not shown).

The majority of the stimulatory activity in GCCM fractionated as described for STIM2 as opposed to STIM1, suggesting that STIM2 could be a major regulator of pontine afferent elaboration during innervation of the cerebellum. Like STIM1, STIM2 stimulated the extension of long neurites from pontine neurons (>300 μM) while short neurites were produced by granule cells (~ 50 μM; Fig. 5C).

Comparisons of GCCM, STIM1 and STIM2 revealed that all three fractions stimulated pontine neurite extension. However, in cultures treated with STIM1 and STIM2, long neurites were thin and exhibited no axonal spreading as seen in GCCM-treated cultures (Fig. 6A). In addition, branchlet formation was substantially reduced. Thus, STIM1 and STIM2 are separable and distinct from granule cell derived factors that promote axonal spreading such as Wnt7a [11].

3.4. Cooperativity between STIM1 and STIM2

The data presented above show that STIM1 and STIM2 exhibit properties expected of retrograde signaling molecules that are secreted by target neurons to regulate the growth of innervating afferents. It is puzzling that granule cells produce two distinct activities that both induce rapid morphological changes in pontine afferents. However, these two activities might be expressed by granule cells in distinct cerebellar regions, or at slightly different stages of cerebellar development, or used together to elicit a heightened response. To investigate the potential for cooperativity between these two activities, we tested low concentrations of STIM1 and STIM2 separately compared with STIM1 and 2 combined. STIM1 and STIM2 were titrated to within a window of stimulation of 1.5- to 2-fold above background (Fig. 6B). When STIM1 and STIM2 were combined, stimulation exceeded that of either alone (+171 μm versus +80 μm and +37 μm, when corrected for background) and was higher than the additive effect

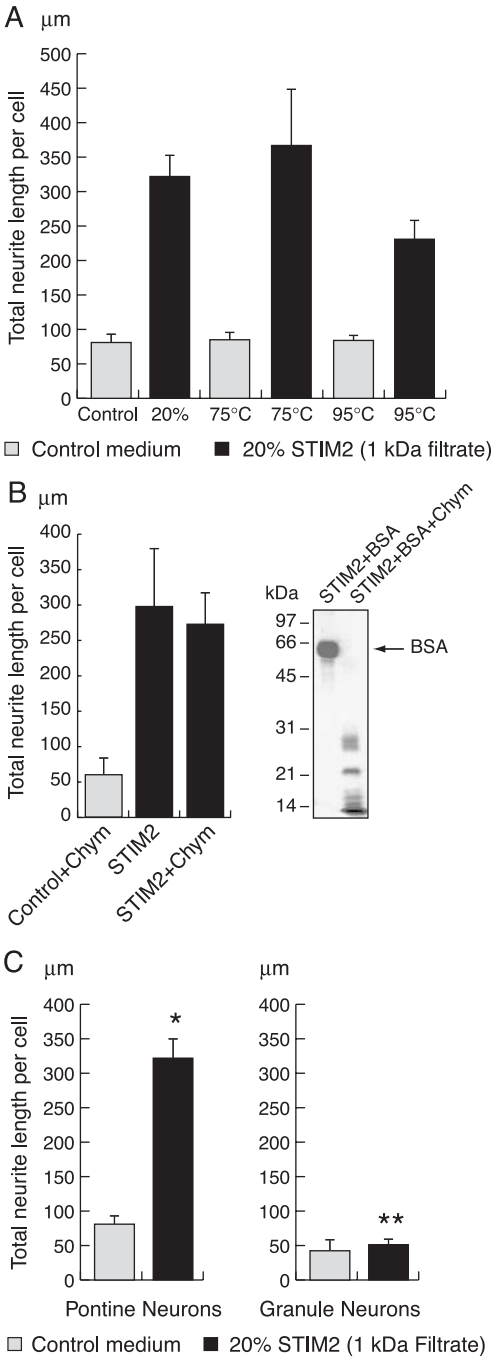

Fig. 5. Analyses of STIM2 activity. (A) Analysis of heat stability of STIM2 activity from 1 kDa BSA-FT fraction. Samples were heated to 75 and 95 °C, respectively, and cleared by filtration through a 1 kDa cutoff membrane. Statistical significance (Student's *t* test, unpaired) for each pair of control medium and STIM2: $p < 0.01$. (B) Proteolytic treatment of STIM2 fraction using chymotrypsin. Samples were incubated at 37 °C for an hour in the presence or absence of 100 μM chymotrypsin, followed by blocking and removal of protease (see Materials and Methods for details). Samples were tested for stimulation of pontine neurite growth at a concentration of 10%. Statistical significance for column 1 versus 3: $p < 0.0005$. No statistical significance ($p > 0.05$) comparing column 2 and 3. As STIM2 fractions do not contain detectable amounts of protein by conventional SDS–PAGE analysis, fractions were supplemented with BSA to verify chymotrypsin activity and analyzed by Coomassie-stained SDS–PAGE. The position of BSA is indicated by the arrow and the migration of a molecular weight standard is shown on the left. (C) To investigate the selective ability of STIM2 to promote the extension of long neurites, assays for neurite outgrowth were performed using pontine neurons (left panel) in comparison to granule neurons (right panel). The assays were performed in parallel under identical conditions. Statistical significance: *$p < 0.0001$ for pontine neurons (Student's *t* test, unpaired). No statistical significance (**$p > 0.05$) for control versus STIM2 with granule neurons. The histograms show the average of three to four experiments and the error bars represent the S.E.M.

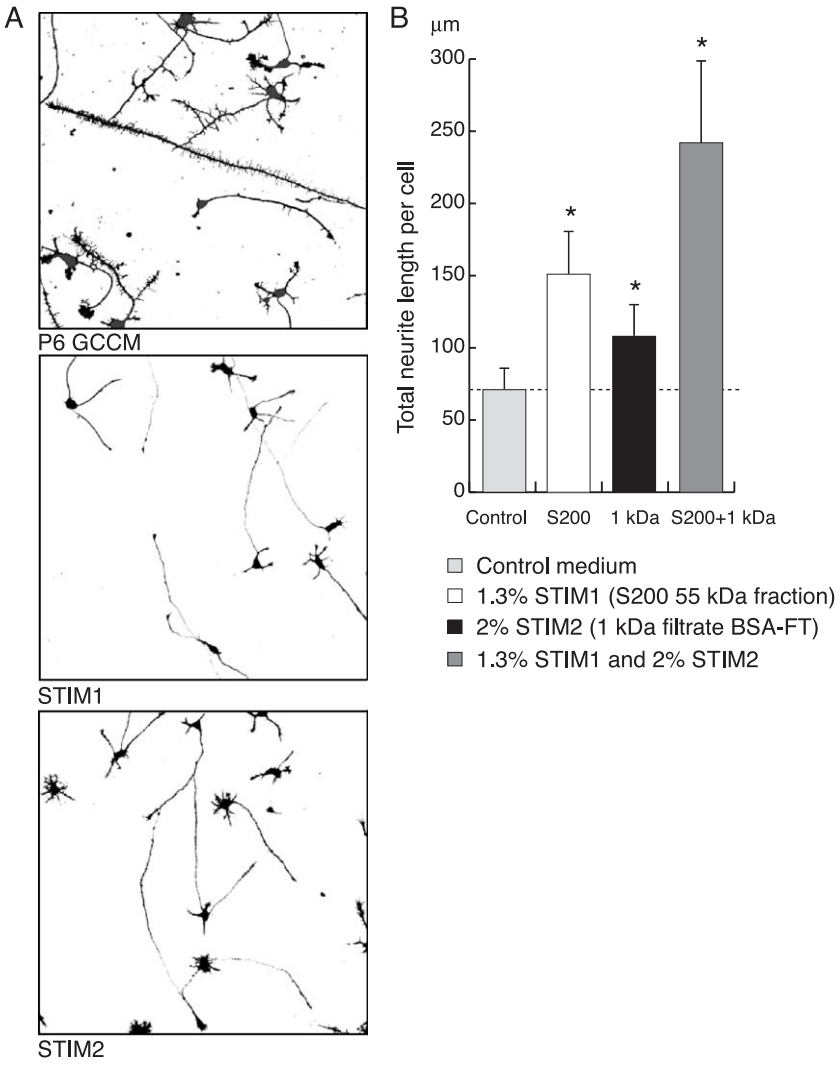

Fig. 6. (A) Comparison of pontine neurites stimulated with 6% GCCM (P6), STIM1 (10% S200 55 kDa fraction), and STIM2 (20% 1 kDa filtrate). (B) Cooperativity between STIM1 and STIM2. Assays for pontine neurite growth were performed using low amounts of STIM1 or STIM2 to achieve intermediate levels of neurite growth. This is compared to stimulation of neurite growth in presence of both STIM1 and STIM2. The values for the four conditions are 71 μm (control, dotted line), 151 μm (STIM1), 108 μm (STIM2), and 242 μm (STIM1 + STIM2). The increase in neurite length above background level (indicated by dotted line) in presence of both STIM1 and STIM2, 171 μm, is more than the sum of the increases above background for STIM1 and STIM2, 117 μm (80 + 37 μm), when assayed separately indicating that there is positive cooperativity between STIM1 and STIM2. Statistical significance (two-way ANOVA): $*p < 0.005$ that columns 2, 3, and 4 have the same mean. The data show the average of four experiments and the error bars represent the S.E.M.

(171 μm versus 117 μM) (see Fig. 6 for details). Thus, STIM1 and STIM2 show positive cooperativity in stimulating pontine neurite growth, and could potentially work together to have a stronger impact on neurite extension than either one alone.

4. Discussion

Pontine neurons are known to form elaborate interactions with cerebellar granule neurons. However, the signals that regulate the development and maturation of pontine arbors are not fully understood. Using a new bioassay system, we provide evidence that target-conditioned medium can have profound effects on the growth and morphology of presynaptic neurites in vitro. Activities derived from cerebellar or granule cell conditioned medium have specific and acute stimulatory effects on pontine neurite growth and morphology. These effects are evident in a low-density culture system indicating that stimulation is independent of homo- or heterotypic cell–cell contacts. Through biochemical fractionation, we have found that growth stimulation is largely attributable to two distinct activities. Experiments using a panel of candidate factors have not revealed their identity, although complex combinatorial interactions of multiple candidate factors cannot be ruled out. Taken together, our data suggest that GCCM contains as yet unidentified factors, described herein as STIM1 and STIM2, that act directly rather than through an indirect or autocrine pathway to stimulate pontine neurite growth.

4.1. Bioassay development and effects of GCCM

The format of our bioassay system was optimized to investigate the existence of diffusible, target-derived activities that influence the development of presynaptic afferents. In contrast to previous analyses of interneuronal signaling, we utilized a low-density culture system in order to minimize the impact of cell–cell contacts and the fasciculated growth of nearby neurites. Most analyses of diffusible interneuronal factors have used co-cultures, tissue explants, or high density dissociated cultures to ensure neuronal survival in long-term cultures. Since our bioassay was complete within 24 h, we could employ a low-density culture system with 75% survival. In addition, this assay utilized a highly permissive substrate to assay for factors that stimulate neurite extension per se rather than factors that alter the substrate. Finally, our assay was designed to detect activities that promoted neurite growth in a non-directional manner.

This sensitized bioassay allowed us to detect two distinct activities in GCCM that rapidly influence pontine neurite outgrowth, even in the absence of BDNF. We attribute granule cells to be the primary source of these activities. However, while the presence of stimulatory activity clearly correlates with the presence of granule cells, we cannot exclude the possibility that small amounts of other cell types (~ 1% GFAP positive, other unconfirmed neuronal cell types) present in our GCCM cultures might produce factors that antagonize or synergize with STIM1 or -2, although few other neuronal cell types are sufficiently abundant from P0 through P14. For instance, Purkinje cells have been reported to produce Sema3A that causes growth cone collapse of pontine axons [28] and could antagonize stimulation by STIM1 or -2. This could potentially explain the low level of stimulation seen with the large cell fraction, which is enriched for Purkinje cells and thereby could antagonize stimulation by granule cells contaminating that fraction. Indeed, such competing outgrowth-promoting and inhibiting factors could restrict pontine elaboration to the boundary below the Purkinje layer.

In addition to the effects on neurite outgrowth, these bioassays revealed that CCM and GCCM had a stimulatory effect on branchlet formation in pontine neurons. Interestingly, branchlets were most prevalent on presumptive axons, but similar structures were also observed on presumptive dendrites with less frequency and with a shorter, more stubby appearance. In addition, branchlets on long presumptive axons tended to be more distal to the cell soma, and longer presumptive axons developed bona fide branches. We speculate that these branchlets may be precursors to bona fide branches. It will be interesting to elucidate the nature of these branchlets and to determine the role of STIM1/2 in branchlet formation once these factors have been identified conclusively and can be manipulated in pure form.

4.2. Characterization of STIM1/2 and candidate molecules

A large and diverse set of molecules that affect neurite growth and morphology has been identified through cell-based and biochemical assays or genetic screens [16,36]. Studies by Rabacchi et al. [27] shed light on the effects of cerebellar BDNF, NT4/5, and other neurotrophins on pontine neurite outgrowth. In addition, Maxillary Factor, a long-sought trigeminal chemoattractant, was found to consist of the combined effect of BDNF and NT-3 [24]. We therefore tested a range of neurotrophins, both singly and in combination with BDNF, for their ability to recapitulate the effects of GCCM. When screening fractionated GCCM, our sensitized assay included BDNF as a standard media component, leaving open the possibility to identify factors dependent on the trophic support of BDNF. In the end, we could not find a known neurotrophin to substitute for the effects of STIM1/2, and we found that the active fractions containing STIM1/2 were free of BDNF and stimulated neurite outgrowth even in culture medium lacking BDNF.

Another class of molecules known to affect cerebellar neurite outgrowth and morphology are the extracellular matrix (ECM) and cell adhesion molecules (CAM) [5,13,31]. L1 is of particular interest, as it has been shown to be expressed by cultured granule cells [18] and to stimulate granule neurite outgrowth [7]. However, STIM1/2 cannot be identical to L1, as the soluble fragment of L1 is 140 kDa, considerably larger than STIM1/2. Potentially, STIM1 could be a degradation product of L1, but we find that to be highly unlikely since GCCM contains substantial amounts of STIM1 and yet fails to stimulate GC neurite outgrowth as previously reported for L1 [7]. In general, adhesion molecules tend to be much larger than STIM1/2 and, thus, we find it unlikely that STIM1/2 could be an adhesion molecule, although we cannot exclude the possibility that a CAM or ECM fragment could contribute to the effects of STIM1/2.

Results published by Salinas et al. [11,20] are consistent with our observations that GCCM increased pontine neurite area and branchlet formation. In their studies of Wnt-7a as a synaptogenic factor, they observed that postnatal day 1 (P1) pontine explants respond to either GCCM or heterologous cells expressing Wnt-7a with an increase in axonal complexity and filopodia formation. Although these results are in keeping with those presented here, the appearance of Wnt-7a occurs several days after (at P6) the appearance of STIM1/2 activities, whose production is seen as early as P0. Thus, STIM1/2 activity cannot be attributed to Wnt7a. Furthermore, the effects of GCCM on neurite extension and branchlet formation are separable, as partially purified STIM1/2 activities stimulate neurite elongation without an effect on branchlet formation. Identification of these outgrowth-promoting activities will be important to understand the relative contributions of factors present in GCCM and how the timing of each factor's production is relevant for neurite growth and maturation.

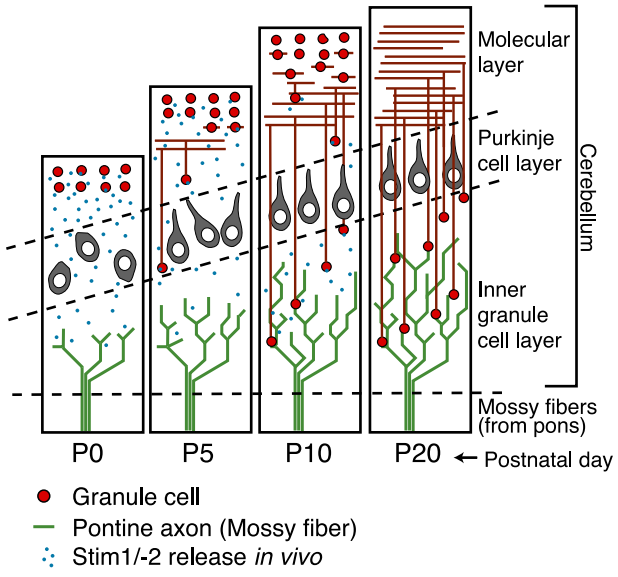

Fig. 7. Model for stimulation of pontine afferent arborization by STIM1/2 in vivo. Active fractions of STIM1/2 can be isolated from GCCM collected from P0 to P14 mice. The timing of STIM1/2 production and release coincides with the period of pontine afferent growth and arborization in the inner granule layer. We propose that STIM1/2, produced by granule neuron precursors and/or maturing granule neurons, constitute target-derived cues that stimulate pontine afferent maturation prior to synapse formation. (Figure adapted from Ref. [13].)

The biochemical characteristics of STIM1 and STIM2 fit the profiles of a polypeptide and a small molecule, respectively. In the case of STIM2, discerning its approximate size and stability suggested a new range of molecules to test as potential candidates. Adenosine, guanosine, ATP and GTP have been suggested to enhance neurite outgrowth and have been proposed to stimulate axonal regeneration following brain injury [9,10,29]. L-Serine and glycine have been reported as trophic factors for Purkinje neurons, but these are produced primarily by glial cells, unlike STIM1/2 that are produced primarily by granule cells [8]. However, none of the small molecules that we tested appeared able to elicit the stimulatory effects observed with STIM2.

4.3. Potential role of STIM1 and STIM2 in cerebellar development

The postnatal production of the active components of GCCM is robust, beginning at P0 and continuing at declining levels to P14. After that age, cerebellar granule cells do not survive well in culture, raising concerns about the interpretation of subsequent experiments. The steady decline of stimulatory activity in CCM per milligram cerebellum suggests that the in vivo production of STIM1 and STIM2 may be developmentally regulated. Interestingly, STIM1/2 are present several days prior to Wnt7a, suggesting that these factors may act in a timely coordinated fashion to promote pontine neurite maturation.

Given that pontine fibers innervate the cerebellum prior to population of the granule cell layer, interactions between pontine fibers and granule cells must occur through diffusible cues. We propose that STIM1/2 could constitute such diffusible cues and hypothesize that granule cell precursors and/or migrating granule cells secrete STIM1/2, which could then diffuse past the Purkinje cell layer to affect pontine afferents in the inner cerebellar layer as they await the maturation of their granule cell targets (Fig. 7). Thus, STIM1/2 could stimulate pontine afferent elaboration during the time period following pontine afferent arrival and before the initiation of synaptogenesis that is stimulated by factors such as Wnt-7a.

Acknowledgements

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