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Semaphorin-3F attracts the growth cone of cerebellar granule cells through cGMP signaling pathway

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Abstract

Growth cone extension is guided by extracellular factors during the brain development but the underlying cellular mechanisms remain largely unclear. Here, we examined the potential function of class-3 semaphorins in cultured cerebellar granule cells. We found neuropilin-2 (NP2), the high-affinity receptor for semaphorin-3F (Sema3F), is highly expressed in cerebellar granule cells. An extracellular gradient of Sema3F triggered an NP2-dependent attractive turning of the growth cone of cultured cerebellar granule cells. This Sema3F-triggered growth cone attraction was abolished by inhibition of the cGMP signaling pathway and reduced by elevating the intracellular cGMP level. Furthermore, Sema3F partially rescued the collapse induced by inhibition of basal cGMP in granule cells. Thus, Sema3F may act as a chemoattractant for the growth cone of cerebellar granule cells through cGMP signaling pathway.

Keywords: Semaphorin; Growth cone guidance; Turning assay; Neuropilin; cGMP; cAMP; Granule cell; Development; Cerebellum

The wiring of neuronal circuits during brain development depends on the guidance of growth cone extension by extracellular factors [1]. Some growth cone guidance molecules have been discovered and their receptors have been identified [2]. The same guidance factor can trigger either attraction or repulsion to growth cones of different neurons and to the same group of neurons at different developmental stages [2,3]. It has been suggested that the growth cone's response to the gradient of a guidance factor can be "switched" by changing the level of intracellular cyclic nucleotides, either cAMP or cGMP, with high levels of cyclic nucleotide promoting the growth cone attraction in response to the factor and low levels promoting repulsion [3].

Among several conserved families of axon guidance molecules, semaphorin (Sema) belongs to one of the largest families. Both secreted and membrane-bound semaphorins have been identified. They were divided into seven classes (Semaphorin Nomenclature Committee, 1999). Semaphorin-3A (Sema3A) and Semaphorin-3F (Sema3F) are two extensively studied members of the class-3 secreted semaphorins in vertebrates, both of which play important roles in brain development as potent chemorepellants to axonal extension and neuronal migration [4]. They propagate the intracellular signaling through the receptor complex of neuropilin and plexinA proteins [4]. Studies in cultured *Xenopus* spinal neurons showed that growth cone turning triggered by Sema3A can be "switched" through changing the intracellular cGMP level [5]. However, Sema3A-triggered growth cone collapse in dorsal root ganglion (DRG) neurons was suppressed by elevating the cAMP level [6]. These studies support a modulatory effect of cyclic nucleotides to the guidance effect of semaphorins. However, whether cyclic nucleotides can directly mediate the growth cone guidance signal of extracellular factors remains unclear.

In the present study, we examined the potential role of the two members of class-3 semaphorins, Sema3A and Sema3F, in the guidance of neurite extension of cultured

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cerebellar granule cells of rat. We found that both Sema3A and Sema3F are expressed in the developing cerebellar tissue, while only NP2, the receptor for Sema3F, is expressed in cerebellar granule cells. Surprisingly, Sema3F acted as an attractant rather than a repellant to cultured granule cells. Moreover, cGMP signaling pathway was essential for Sema3F-triggered growth cone attraction.

Materials and methods

Cell culture. Cerebellar tissues of neonatal Sprague–Dawley rats were incubated in CMF-PBS (calcium- and magnesium-free PBS) containing 0.1% trypsin (Sigma, St. Louis, MO) for 8 min at 37 °C, followed by trituration. Dissociated cells were collected by centrifugation, resuspended, and plated on coverslips coated with Laminin (25 μg/ml, Sigma), and cultured in Neurobasal Medium supplemented with B27 (Gibco, Grand Island, NY) and fetal bovine serum (FBS, PAA, Pasching, Austria) as described before [7,8]. Cells were used for the turning assay after 17 h incubation.

Plasmids and siRNA transfection. The cDNA for Sema3F-myc was a gift from M. Tessier-Lavigne. The NP2 RNAi was constructed by cloning fragment of 5′-gctatgacatggagtatca-3′ into the pSuper vector (OligoEngine, Seattle, WA). For plasmid transfection, we used the Rat Neuron Nucleofector Kit (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, dissociated cells were resuspended in transfection medium, mixed with plasmids (6 μg), and electroporated using the fixed programme (O-03) [7] for optimal neuronal transfection. Cells were then quickly centrifuged, resuspended and plated. The knockdown efficiency was determined by Western blotting of extracts from transfected cultures.

Explant culture and co-culture. Neonatal brains were dissected out, selected parts of cerebellum (mainly EGL) were dissected using fine tungsten needles to extract small tissue pieces (approximately 200–300 μm diameter). Co-cultures consisted of different combinations between the cerebellum explants and aggregates of control or Sema3F-, Slit-2-expressing HEK 293 cells. Explants were embedded in matrigel or a mixture of matrigel and collagen (BD Biosciences, Bedford, MA) as described previously [9], and cultured for 48–72 h in Neurobasal medium supplemented with B27. Cell quantification was performed after fixation with 3% paraformaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer (PB) and stained with propidium iodide (PI).

Collapse assays. Hippocampus and cerebellum explants were placed on glass coverslip coated with Lamina (25 μg/ml) and cultured in Neurobasal medium supplemented with B27 and FBS. After 18 h (cerebellum) or 48 h (hippocampus) incubation, 40 μl of concentrated control medium or conditioned media (without FBS) containing different recombinant semaphorins were added into the culture for 30 min. Explants were then fixed as described above, and observed by confocal microscope (FV2000, Olympus). In each explants, we counted the number of fan-like growth cones with abundant lamellipodia and filopodia and "collapsed" growth cones with round-tipped or pencil-like morphology [10].

Growth-cone turning assay. Microscopic gradients of guidance factors were produced as described previously [5,7,11]. The conditioned media in the micropipette was ten times concentrated. Images of neurites were recorded and analyzed using the program Scion Image 4.0.2. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the growth-cone positions at the onset and the end of the 1-h period. Pharmacological reagents were applied 30 min before the turning assay and presented throughout the assay. Rp-8-Br-cAMPs, Rp-8-Br-cGMPs, SP-8-Br-PET-cGMPs, and KT5823 were from Calbiochem. KT5720 was from Sigma.

RT-PCR and Western blotting. For reverse transcriptase polymerase chain reaction (RT-PCR), total RNA was isolated from cerebellum tissue and cultures of cerebellar granule cells with Trizol reagent (Invitrogen, Carlsbad, CA). About 5 μg of total RNA was converted to cDNA (RevertAid First Strand cDNA Synthesis Kit, Fermentas UAB, Vilnius, LT), and 0.2 μl of the cDNA was used in 20-μl PCRs. Primers and conditions for the PCR were listed in Table 1. End reaction products were visualized on ethidium-bromide-stained 1.5% agarose gels. To detect the expression levels of NP1 and NP2, tissues or cultured cerebellar granule cells were harvested by lysis buffer (0.1% SDS, 1% nonidet P-40, 1% glycerin, 50 mM Hepes, pH 7.4, 2 mM EDTA and 100 mM NaCl). Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membrane. Blots were blocked for 3 h at room temperature in 5% BSA, incubated with a polyclonal antibody (1:500, R&D, Minneapolis, MN) overnight at 4 °C, rinsed and incubated for 1 h at room temperature with a horseradish-peroxidase-conjugated goat antibody against rabbit or mouse IgG (1:10,000; Bio-Rad, Quarry Bay, Hong Kong). Chemiluminescence detection was performed with the ECL kit (Pierce, Rockford, IL).

Results

Sema3A and Sema3F do not trigger growth cone collapse in granule cells

In order to test the potential function of secreted semaphorins to cerebellar granule cells, we first examined the expression of Sema3A and Sema3F as well as their receptors in the cerebellar tissue of neonatal rats by RT-PCR. As a positive control, we examined in parallel the hippocampal tissue, which is known to express these factors and receptors [12]. As shown in Fig. 1A, the mRNAs for both Sema3A and Sema3F were detected in the cerebellar tissue. The mRNAs for NP1 and NP2, the high-affinity receptors for Sema3A and Sema3F, respectively, were also detected. Western blotting with specific antibodies against NP1 and NP2 were performed to further clarify the expression of these two receptors in the cerebellar tissue. As

Table 1
Conditions for RT-PCR

Object	Primer	T_m (°C)	Length (bp)	Cycles
Sema3A	Sense: 5′-GGCTCCTGCTTCGTAGTCT-3′ Anti-sense: 5′-GTGGGTCCTCCTGTTTCT-3′	50	389	34
Sema3F	Sense: 5′-TCAACAAGTGGAGCACATTC-3′ Anti-sense: 5′-ACAGTGGTGAGGCGGTAG-3′	53	466	39
NP1	Sense: 5′-ATTTCAAGTGTATGGAGGCT-3′ Anti-sense: 5′-AGTAACGAATCGCAGGAG-3′	50	197	34
NP2	Sense: 5′-GAACATTGCCCCTCCCACCAT-3′ Anti-sense: 5′-ATCCGGCCAGACTCCATTCC-3′	61	545	34