visual display. Leaf litter could serve to amplify vibrations of courting males (*3, 18*); thus communication by substrate-coupled stridulation would be particularly effective in this environment.

Differences in courtship behavior are clearly important in maintaining the otherwise incomplete isolation of these two species and hence their genetic integrity. We conclude that *S. rovneri* is an ethospecies, reproductively isolated from its sibling species by its unique courtship behavior.

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17. Recordings were played back into a Tektronix 510 3N recording oscilloscope, kindly loaned by E. S. Kaneshiro. Photographs were taken of the oscilloscope screen.
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Ganglioside Stimulation of Axonal Sprouting in vitro

Abstract. *Bovine brain gangliosides were applied to primary and established neuronal cultures to examine the role of gangliosides in neuronal development. Media containing gangliosides enhanced the degree of axonal elongation exhibited by sensory ganglia neurons and increased the length and number of Neuro-2a neuroblastoma cell processes. Ganglioside-supplemented media caused a twofold increase in ornithine decarboxylase activity in both culture systems. These experiments suggest that gangliosides function as acceptor molecules for growth-promoting substances in embryonic and tumor-derived neurons.*

Gangliosides are cell membrane–associated molecules that play a role in a variety of cellular events, including differentiation (*1–3*), defense (*4*), growth (*3, 5, 6*), regeneration (*7*), and transformation (*7*). The striking changes in the distribution and quantity of gangliosides during cephalogenesis (*8*) suggest that they are important in neuronal development. Since gangliosides are mainly associated with cellular membranes, it has been suggested that they act in the transfer of information across these membranes (*9*). Gangliosides function as receptors on the cell surface for glycoprotein hormones (*10*), interferon (*11*), and serotonin (*12*).

To investigate the role of gangliosides in neuronal development, we exposed primary cultures of sensory ganglia and an established neuroblastoma line to media containing mixtures of bovine brain gangliosides. As evaluated morphologically and biochemically, the ganglioside mixtures enhanced neurite development and metabolic activity of both sensory ganglia and Neuro-2a neuroblastoma cells. Thus it appears that gangliosides mediate development in the nervous system.

Dorsal root ganglia from 8½-day-old chick embryos (White Leghorn) were cultured on collagen-coated cover slips, as double cover slip lying-drop preparations, in medium 199 (Gibco) supplemented with 10 percent heat-inactivated fetal calf serum (Irvine Scientific) or in serum-free HI-WO_5/BA_{2000} medium (International Scientific Industries). Neuro-2a murine neuroblastoma cells (CCL-131, American Type Culture Collection) were maintained in plastic and glass petri dishes with Eagle's minimum essential medium containing Hanks balanced salt solution (Gibco) and supplemented with 10 percent fetal calf serum, 10 mg of

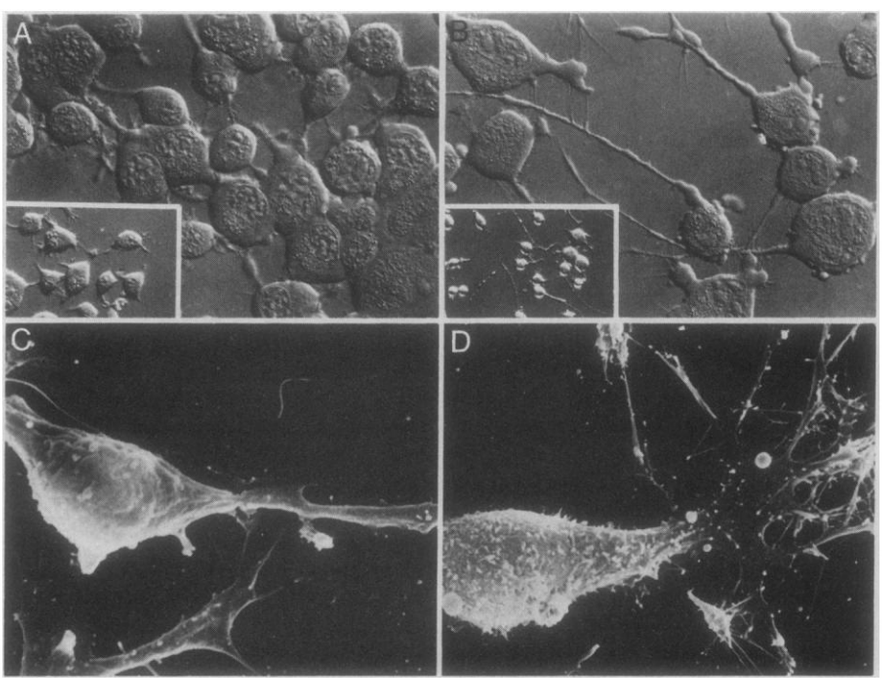

Fig. 1. (A and C) Photomicrographs of Neuro-2a cells grown in control medium for 40 hours. Few processes are seen and the cell surface is relatively smooth, with only occasional microspikes. (A) Nomarski optics (× 300; inset × 130). (C) Scanning electron microscopy (× 1400). (B and D) Photomicrographs of Neuro-2a cells grown for 40 hours in media supplemented with 250 μg of bovine brain gangliosides per milliliter (*13*). Extensive sprouting can be seen on cell surfaces. Numerous microvilli and blebs are also visible on the perikaryon. (B) Nomarski optics (× 300; inset × 85). (D) Scanning electron microscopy (× 1300).

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Table 1. Effect of ganglioside mixture (*13*) on axonal development. Dorsal root ganglia were grown on collagen-coated cover slips in medium with or without gangliosides and observed after 48 hours. Neuro-2a neuroblastoma cells were grown in plastic petri dishes in medium with or without gangliosides and observed after 24 hours. Cultures were coded and randomized to prevent experimenter bias. Data are means ± standard deviations.

Treatment	Dorsal root ganglia		Neuro-2a sprouting index*
	Number of neurites	Length of neurites (μm)	
Medium	50 ± 20	900 ± 100	5 ± 2
Medium + gangliosides (75 μg/ml)	120 ± 30	950 ± 100	30 ± 12
Medium + gangliosides (750 μg/ml)	130 ± 50	750 ± 200	40 ± 15

*Product of length of processes to nearest 10 μm and number of processes per cell; based on 100 cells per treatment group.

gentamicin (Schering) per 100 ml of medium, 75 mg of $NaHCO_3$ per 100 ml of medium, and 0.1 m*M* nonessential amino acids.

The cultures were grown with or without gangliosides (*13*) for 24 or 48 hours in an atmosphere of 5 percent CO_2 and 95 percent air (Table 1). The effects on chick dorsal root ganglia were determined as described by Roisen *et al.* (*14*). The effects on Neuro-2a cells were measured semiquantitatively with high-resolution light microscopy by determining the mean number and length of neurites for a minimum of 100 cells per treatment; these values were multiplied to obtain an index of axonal sprouting.

Gangliosides increased the number of processes originating from sensory ganglia ($P < .05$, Student's *t*-test) (Table 1). Similarly, gangliosides increased the sprouting index value for the Neuro-2a cells ($P < .05$) (Table 1 and Fig. 1, A and B). Time-lapse photography of the Neuro-2a cells showed rapid sprouting within 6 hours following ganglioside exposure. These cells were grown on acid-washed glass, collagen-coated cover slips, and plastic culture surfaces to minimize the possibility that the axonal sprouting index value for the Neuro-2a cells ($P < .05$) (Table 1 and Fig. 1, A and B). Time-lapse photography of the Even the few giant cells in the clonal cultures exhibited dramatic increases in axonal sprouting. In contrast, L-929 murine fibroblasts and S_{20} neuroblastoma cells did not respond morphologically to gangliosides.

Scanning electron microscopy (*15*) revealed that the processes seen under the light microscope underwent morphological changes at their surfaces. Unlike control Neuro-2a cells, whose surfaces were smooth, ganglioside-treated cells had elaborate spine-like projections (Fig. 1, C and D).

A biochemical index of the effects of exogenous gangliosides on growth was obtained by determining the activity level of ornithine decarboxylase (*16*) in dorsal root ganglia and Neuro-2a cells after exposure to gangliosides for 6.5 hours (Table 2). The gangliosides produced a twofold increase in the levels of enzyme activity in both cell types.

Previous studies in our laboratory (*17*) showed that administered gangliosides are incorporated nonselectively into the membranes of primary chick dorsal root ganglia and Neuro-2a neuroblastoma cells. The ability of exogenous gangliosides to enhance axonal sprouting in primary embryonic neurons and established neuronal cultures suggests that these molecules play a key role in regulating neuronal maturation. The ganglioside GM_1 has been shown to stimulate the formation of neuromuscular junctions in vitro (*2*) and to bind cholera toxin (*5, 18*). Furthermore, when normally unresponsive GM_1-deficient fibroblasts are grown in GM_1-containing media, they respond to the toxin (*5, 18*). Cholera toxin increases intracellular levels of adenosine monophosphate as well as substrate adhesion and process formation in rat adrenal pheochromocytoma (PC-12) cells (*19*). Fishman *et al.* (*5*) suggested that the binding of toxin to a specific ganglioside receptor and the subsequent activation of adenylate cyclase provides a model for neurotrophic interactions. Our results are in accord with this notion and suggest further that gangliosides function as acceptor molecules for growth-promoting substances on embryonic neurons.

Table 2. Effect of ganglioside mixture (*13*) on activity levels of ornithine decarboxylase. Abbreviations: SFM, serum-free medium; SDM, serum-depleted medium.

Culture	Treatment	Enzyme activity (counts/mg-protein)
Dorsal root ganglia	SFM	1600
	SFM + buffer	1300
	SFM + gangliosides (750 μg/ml)	3100
Neuro-2a	SDM	1300
	SDM + buffer	900
	SDM + gangliosides (750 μg/ml)	2400

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