Glial Lipoproteins Stimulate Axon Growth of Central Nervous System Neurons in Compartmented Cultures

Hideki Hayashi1,4, Robert B. Campenot2, Dennis E. Vance3,4 and Jean E. Vance1,4

Departments of 1Medicine, 2Cell Biology and 3Biochemistry, and 4CIHR Group on Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Correspondence to:
Jean E. Vance
Group on Molecular and Cell Biology of Lipids
328 HMRC
University of Alberta
Edmonton
AB, Canada T6G 2S2

Phone: (780) 492-7250
Fax: (780) 492-3383
email: jean.vance@ualberta.ca

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SUMMARY

The role of lipoproteins secreted by cortical glial cells in axon growth of central nervous system (CNS) neurons was investigated. We first established compartmented cultures of CNS neurons (retinal ganglion cells). Addition of glial cell-conditioned medium (GCM) to distal axons increased the rate of axon extension by ~50%. Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase in glial cells diminished the secretion of cholesterol and apolipoprotein E, and prevented the growth stimulatory effect of GCM. When glia-derived lipoproteins containing apolipoprotein E were provided to distal axons, axon extension was stimulated to the same extent as by GCM. In contrast, addition of lipoproteins to cell bodies failed to enhance growth. The growth stimulatory effect of glial lipoproteins was abrogated in the presence of receptor-associated protein, RAP, indicating involvement of receptor(s) of the low density lipoprotein receptor family in stimulation of axonal extension. These observations suggest that glial cells stimulate axon growth of CNS neurons by providing lipoproteins containing cholesterol and apolipoprotein E to distal axons.
Identification of factors that regulate axon growth and regeneration of CNS neurons has been hampered by the lack of a model that recapitulates the in vivo environment. In intact animals, neuronal cell bodies and axon terminals often reside far apart in distinct environments. The issue of spatial separation of cell bodies and axons was addressed for peripheral neurons by the establishment of sympathetic neuron cultures in which cell bodies were plated in one compartment and axons extended into adjacent compartments (1). Consequently, cell bodies and distal axons could be exposed to independent fluid environments and the rate of axon extension accurately measured. Our objective was to culture CNS neurons in compartmented cultures and identify factors that stimulated axon growth.

The most numerous cell types in the brain are glial cells: oligodendrocytes, astrocytes and microglia. Glial cells interact with neurons and are associated with neuronal excitability and synaptic transmission (2) (3-7). Glial cell-conditioned medium (GCM) stimulates synapse development of CNS neurons (8,9), and cholesterol, associated with apolipoprotein (apo) E, was identified as the factor that promotes synaptogenesis (10).

Apo E is synthesized in the brain primarily by astrocytes and microglia (11-13) and is the major CNS apolipoprotein. Apo J is also abundant in the brain (14). Cerebrospinal fluid, which is separated from the plasma compartment by the blood-brain barrier, contains a population of lipoproteins distinct from those in plasma (12,15-18). Several observations imply that apo E plays an important role in lipid metabolism in the nervous system (19). For example, apo E synthesis in glial cells dramatically
increases after injury to the central (20-22) and peripheral nervous systems (21). Apo E is also a ligand for receptors of the low density lipoprotein receptor (LDLr) superfamily, several of which (e.g. LDLr, LDLr-related protein, very low density lipoprotein receptor and apo E2 receptor) are expressed in CNS neurons (23). The three most common apo E alleles in humans are E2, E3 and E4 (24). Inheritance of the apo E4 allele is the strongest known risk factor for late-onset Alzheimer’s disease (25-27). In addition, in various neuronal cell lines, apo E3 either stimulates axon growth or is neutral, whereas apo E4 is either neutral or inhibits growth (28-31). We have successfully established compartmented cultures of CNS neurons for the first time and have demonstrated that apo E-containing lipoproteins secreted by glial cells enhance axon growth of CNS neurons.
MATERIALS AND METHODS

Isolation and culture of rat retinal ganglion cells (RGC) – RGC were isolated from rats by the method of Barres et al. (32), with minor modifications. Briefly, retinal tissue was dissected from one-day-old Sprague-Dawley rats. Twelve retina were combined and incubated for 20 min at 37°C in phosphate-buffered saline containing 16.5 units/ml papain (Worthington, Lakewood, NJ). Tissues were triturated in phosphate-buffered saline containing 0.15% trypsin inhibitor (Roche, Mannheim, Germany), 0.15% bovine serum albumin (Sigma) and rabbit anti-macrophage antiserum (Accurate Chemical and Scientific Corp., Westbury, NY) until retinas were dissociated. Cells were pelleted by centrifugation for 5 min at 200 x g then suspended in phosphate-buffered saline containing 1% trypsin inhibitor and 1% bovine serum albumin. The retinal cell suspension was centrifuged at 200 x g for 5 min and the pellet was resuspended in panning buffer (phosphate-buffered saline containing 0.02% bovine serum albumin and 5 µg/ml insulin). Macrophages and microglia were removed by incubation of the cell suspension for 40 min at room temperature on a panning plate (150 mm Petri dish) coated with goat anti-rabbit IgG. Medium containing the non-adherent cells was filtered through a 15 µm Nitex filter (SEFAR America Ltd., Kansas City, MO) to remove cell aggregates. The filtrate was incubated for 1 h at room temperature on a second panning plate (100 mm Petri dish) coated with goat anti-mouse IgM µ-chain specific antibody (Pierce) and mouse anti-Thy1.1 antibody (specific for RGC) generated from T11D7e2 cells (AmericanType Culture Collection). The panning plate was washed 8 to 10 times with phosphate-buffered saline. Adherent cells were released from the
panning plate by incubation with 0.125% trypsin in phosphate-buffered saline for 8 min at 37°C. Fetal bovine serum (30%) in Neurobasal medium (Gibco, Grand Island, NY) was added to the suspension and RGC were collected by centrifugation at 200 x g for 10 min. The pellet was then resuspended in base medium [Neurobasal medium containing glutamine (2 mM), insulin (5 µg/ml), N-acetylcysteine (60 µg/ml), progesterone (62 ng/ml), putrescine (16 µg/ml), sodium selenite (40 ng/ml), bovine serum albumin (0.1 mg/ml), triiodothyronine (40 ng/ml), transferrin (0.1 mg/ml), sodium pyruvate (1 mM) and 10 µM forskolin (Sigma)] to which were added 25 ng/ml brain-derived neurotrophic factor (PeproTech Inc., Rocky Hill, NJ), 50 ng/ml ciliary-derived neurotrophic factor (PeproTech Inc., Rocky Hill, NJ), 50 ng/ml basic fibroblast growth factor (PeproTech Inc., Rocky Hill, NJ), 2% B27 (Gibco, Grand Island, NY) (32), as well as 0.2% methylcellulose (33).

Culture dishes (35 mm) were coated with poly-D-lysine (Sigma) and laminin (Sigma) (32). Twenty parallel tracks were made by scratching the surface substratum of the dishes (33). The scratched area was wetted with base medium containing the growth factors listed above. A Teflon divider (width ~ 0.5 mm), which creates 3 compartments (33), was sealed to the dish with silicone grease applied from a 20-gauge needle. Isolated RGC were plated in the center chamber of the compartmented dishes at a density of 10,000 to 12,000 cells/dish in base medium containing the growth factors listed above. The side compartments contained the same medium as the center compartment except that basic fibroblast growth factor was omitted and the concentration of brain-derived neurotrophic factor was increased to 75 ng/ml. Within 5 to 7 days, axons crossed under the silicone grease barriers into the left- and right-side
compartments. Medium was changed every 3 days and neurons remained viable for at least 3 weeks.

Measurement of axon extension – Distal axons were mechanically removed (axotomized) from the side compartments of 10-day-old RGC with a jet of sterile water delivered with a syringe through a 22-gauge needle. The wash was repeated 3 times after which fresh medium was added. Extension of axons was measured as the length of the farthest extending axon using a Nikon Diaphot inverted microscope equipped with a MD2 microscope digitizer (Accustage Corp. Minneapolis, MN) (33).

Culture of glial cells – Glial cells were isolated from one-day-old rats (14). Briefly, the cerebral cortex was digested by trituration in phosphate-buffered saline containing 0.25% trypsin. Dissociated glial cells were cultured in 75 mm² flasks (Falcon, BD Biosciences, Bedford, MA) in Dulbecco's modified Eagle’s medium containing 10% fetal bovine serum. After 7 to 9 days, glial cells were harvested from the dish by treatment with 0.125% trypsin, then replated and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum until use (< one month). The glial cell preparation was highly enriched in astrocytes (90.3%; total number of cells = 865) as determined by staining the cell preparation with antibodies directed against glial fibrillary acidic protein (BD Biosciences, Bedford, MA) as a marker of astrocytes, and with Hoechst dye as a stain for nuclei.
Isolation of lipoproteins from glial-conditioned medium – The culture medium was removed from the glial cells, which were then washed 3 times with phosphate-buffered saline, after which base medium (BM) was added. Conditioned medium was collected from glial cells that had been cultured in BM for 3 days (34). The medium was centrifuged for 10 min at 1,000 x g to remove cell debris. The supernatant was designated glial cell-conditioned medium (GCM). Lipoproteins were isolated from GCM on a discontinuous sucrose gradient (14) consisting of the following layers of sucrose solutions: 2 ml of density 1.30 g/ml; 3 ml of density 1.20 g/ml; 3 ml of density 1.10 g /ml; 4 ml of culture medium of density 1.006 g/ml. The gradient was centrifuged in a SW40-Ti rotor (Beckmann) at 4°C for 48 h at 160,000 x g. Twelve, 1.0 ml fractions were removed from the top of the tube and subjected to immunoblotting as described below. The density of each fraction was determined as the weight of 1.0 ml. Fractions containing apo E were identified (typically fractions 5 to 8), then combined, washed 3 times, and concentrated in Neurobasal medium (Gibco, Grand Island, NY) using an Amicon Ultra filter (100 kDa molecular weight cut-off; Millipore, Bedford, MA). The cholesterol concentration was adjusted to 1 µg/ml with base medium. For measurement of the cholesterol content, the concentrated lipoprotein fraction was extracted twice with hexane/isopropanol (3:2, v/v). The solvents were evaporated and bis(trimethylsilyl)trifluoroacetamide was added for 20 min at 50°C to generate the trimethylsilyl derivative of cholesterol. The solvent was evaporated and the sample was resuspended in hexane for analysis by gas-liquid chromatography (35).

For some experiments, lipoprotein-depleted GCM was prepared. GCM (5.0 ml) was gently mixed for 30 min with 50 mg Cab-o sil [colloidal silica that avidly binds
lipoproteins (purchased from Sigma) (36)]. The Cab-o-sil, which contained bound lipoproteins, was removed by centrifugation for 5 min at 1,000 \( \times \) \( g \). The Cab-o-sil-treated GCM contained no detectable apo E or apo J according to immunoblotting with anti-apo E antibodies and anti-apo J antibodies, as described below.

In some experiments, recombinant rat receptor-associated protein [RAP; from PROGEN (Heidelberg, Germany)] was added to the lipoprotein preparation.

*Immunocytochemistry* – The Teflon divider was removed from compartmented cultures of RGC and cells were washed 3 times with phosphate-buffered saline. The RGC were then fixed by treatment with 4% paraformaldehyde for 20 min, permeabilized in 0.2% Triton X-100 for 10 min, and blocked with 5% goat serum in phosphate-buffered saline for 1 h. The neurons were incubated for 16 h at 4°C with mouse monoclonal anti-bovine tau-1 antibody (dilution 1:750; Chemicon, Temecula, CA) and rabbit polyclonal anti-rat MAP2 antibody (dilution 1:1,000, Chemicon, Temecula, CA) in phosphate-buffered saline containing 2% bovine serum albumin. The RGC were washed 3 times with phosphate-buffered saline then incubated with Texas Red-conjugated goat anti-mouse IgG (dilution 1:200, Molecular Probes, Eugene, OR) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (dilution 1:200, Molecular Probes, Eugene, OR) as secondary antibodies for 1 h at room temperature. The cells were washed 3 times with phosphate-buffered saline and mounted with anti-fade reagent (Molecular Probes, Eugene, OR). Pictures were taken using a Leica DMI RE2 microscope.
**Immunoblot analysis** – Proteins were dissolved in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 5% ß-mercaptoethanol. Proteins were separated by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% SDS, then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with goat polyclonal anti-human apo E antibody (dilution 1:1,500, from Biodesign, Saco, ME) for 16 h at 4°C, and subsequently with peroxidase-conjugated mouse anti-goat IgG antibody (dilution 1:5,000, from Pierce, Rockford, IL) for 1 h at room temperature. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

**Rat sympathetic neurons** – Neurons were dissociated from the superior cervical ganglia of one-day-old Sprague-Dawley rats and cultured in compartmented culture dishes (37,38).

**Statistical analysis** – Data were compared using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison, unless otherwise noted.
RESULTS

Establishment of compartmented cultures of retinal ganglion cells

We selected retinal ganglion cells (RGC) as suitable CNS neurons for compartmented cultures because these neurons produce long axons in vivo and are a well-characterized type of CNS neuron. RGC were isolated from retinas of one-day-old rats using the “panning” method of Barres et al. (32). Culture dishes (35 mm) were coated with poly-D-lysine and laminin and were divided into 3 compartments by a Teflon divider that was sealed to the dish with silicone grease (33). The neurons (10,000 – 12,000 cells/dish) were plated in the center compartment and produced abundant axon growth into distal compartments within 5 to 7 days, providing the opportunity to measure the rate of axon extension and perform biochemical analyses in studies analogous to those using compartmented cultures of sympathetic neurons (33,35,38-40). The axons extended at a rate of ~0.5 mm/day into side compartments that contained medium supplemented with growth factors (brain-derived neurotrophic factor and ciliary-derived neurotrophic factor) (Fig. 1). The center compartment housed cell bodies and proximal axons (stained with anti-Tau-1 antibody, red), as well as dendrites (stained with anti-MAP2 antibody, green), whereas the two side compartments contained distal axons alone (stained with anti-tau-1 antibody, red) without cell bodies or dendrites. The neurons, which can be maintained under defined conditions for several weeks, were usually cultured for ~10 days prior to the start of the experiments.

Glial cell-conditioned medium promotes axon extension
To determine if factors released by glial cells stimulate axon growth of RGC we cultured glial cells from the cerebral cortex of one-day-old rats (14) for 3 days and collected the glial cell-conditioned medium (GCM) (34). Distal axons were removed (axotomized) from side compartments of the RGC cultures then GCM, or base medium with or without growth factors, was added to the side compartments. Axon extension was measured each day for 4 days. Addition of GCM to regenerating axons increased the rate of axon extension by ~50% compared to that of base medium with or without growth factors (Fig. 2A). In contrast, when GCM was added to the center compartment that contained cell bodies with proximal axons, the rate of extension of distal axons was the same as when base medium, with or without growth factors, was added (data not shown). Furthermore, when base medium was supplemented with rat serum (1 µg cholesterol/ml) or fetal bovine serum (1 µg cholesterol/ml), and supplied to distal axons of RGC, axon extension was the same as that of cells cultured in base medium alone (Fig. 2B).

**Inhibition of hydroxymethylglutaryl-CoA reductase in glial cells abrogates the ability of GCM to stimulate axon extension**

Since glial cells secrete cholesterol-containing lipoproteins, we investigated whether or not lipids and/or lipoproteins released by glial cells were responsible for the observed stimulation of axon growth. Glial cells were cultured in the presence of various concentrations (0 to 1,000 nM) ofcompactin which inhibits the biosynthesis of cholesterol and non-sterol metabolites of mevalonate at the step catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (41). The culture medium was collected and
the amount of apo E and apo J in the medium determined by immunoblotting. The cholesterol concentration of the medium was measured by gas-liquid chromatography. Compactin inhibited the secretion of apo E (Fig. 3A) and cholesterol (Fig. 3B) from glial cells in a concentration-dependent manner, but did not inhibit apo J secretion (Fig. 3A). Because lipoprotein particles containing apo J are relatively lipid-poor compared to those containing apo E- (12,42) we speculate that the secretion of apo J from glial cells might not be sensitive to inhibition of the synthesis of cholesterol and/or non-serol metabolites of mevalonate. Incubation of glial cells with various concentrations of compactin attenuated the ability of GCM derived from the compactin-treated cells to stimulate axon extension, in a dose-dependent manner (Fig. 3C). However, neither cholesterol nor apo E3 alone, supplied at concentrations equivalent to those in GCM, enhanced axon extension (Fig. 4). Furthermore, fetal bovine serum and rat serum, which contained cholesterol and apo E, failed to stimulate axon growth (Fig. 2B). Consistent with the results of our previous studies in rat sympathetic neurons (43), the addition of 1,000 nM compactin in base medium to distal axons alone did not inhibit axon extension (data not shown). These data suggested that lipoproteins containing cholesterol and apo E might have been the component of GCM that stimulated axon extension.

Apo E-containing lipoproteins promote axon extension of RGC

The CNS contains a spectrum of apo E-containing lipoprotein particles of density in the range of high-density lipoproteins (density 1.06 – 1.21 g/ml). These lipoproteins are distinct from the population of lipoproteins in plasma (18,30,44). Sucrose density
gradient ultracentrifugation of GCM and subsequent immunoblotting revealed that apo E was primarily present in lipoprotein fractions of density 1.07 to 1.12 g/ml (Fig. 5). The apo E-containing fractions were combined and the cholesterol concentration was measured by gas-liquid chromatography. The cholesterol concentration was adjusted to 1 µg/ml (this preparation was designated LP-E), which was the same as that in GCM. When BM was supplemented with LP-E and added to distal axons of RGC, axon extension was stimulated to the same extent (i.e. by ~50% after 4 days) as with GCM (Fig. 6A). However, when cell bodies were incubated with the same concentration of LP-E, axon extension was not increased (Fig. 6B). Moreover, provision of LP-E to distal axons of compartmented cultures of peripheral neurons (rat sympathetic neurons) failed to stimulate axon extension (Fig. 7).

The addition of LP-E to GCM derived from glial cells that had been incubated with 1,000 nM compactin restored the ability of GCM to enhance axon extension (Fig. 8A). As confirmation that the growth stimulatory component in GCM was lipoproteins, the lipoproteins were removed from GCM by incubation with Cab-o-sil, a type of colloidal silica that avidly binds lipoproteins (36). Immunoblotting demonstrated that apo E and apo J were not detectable in the Cab-o-sil-treated GCM (Fig. 8B). Figure 8C shows lipoprotein-depleted GCM was no longer able to stimulate axon extension indicating that the growth stimulatory factor in GCM was, indeed, lipoproteins.

These results indicate that the growth-stimulatory factor released by glial cells is LP-E and that LP-E increases axon extension by acting directly on the distal axons.
Receptor-associated protein (RAP) prevents the GCM-mediated enhancement of axon growth

Apo E-containing lipoproteins are known to bind to receptors of the low density lipoprotein receptor (LDLr) superfamily. Several of these receptors, such as the LDLr itself (45,46), the LDLr-related protein (30,47-49), the apo E receptor 2 (also known as LR7/8B) (46,50-52), the very low density lipoprotein receptor (53) and the LR11 receptor (also known as SorLA) (46,54,55) have been identified in neurons. To determine if lipoproteins in GCM mediated the growth stimulation via receptor(s) of this family, GCM containing the receptor-associated protein, RAP, (which inhibits the binding of ligands to receptors of the LDLr family) was added to distal axons of RGC. Fig. 9 shows that in the presence of RAP, GCM no longer increased the rate of axon extension compared to base medium. Moreover, addition of RAP to base medium did not alter the rate of axon extension. These observations suggest that the growth stimulatory effect of apo E-containing lipoproteins secreted by glial cells is mediated by receptor(s) of the LDLr family.
DISCUSSION

We have, for the first time, established a method for culturing CNS neurons (retinal ganglion cells) in 3-compartmented dishes, and have shown that addition of cortical glial cell-conditioned medium to distal axons of RGC increased the rate of axon extension by ~50%. The growth stimulatory factor in GCM was identified as lipoproteins that contained cholesterol and apo E. In contrast, cholesterol and apo E individually, as well as fetal bovine serum and rat serum, failed to enhance axon extension. The increase in axon growth occurred only when the LP-E was provided to distal axons, but not to cell bodies. The stimulation of axon extension by glial cell-derived lipoproteins appears to be mediated by receptor(s) of the LDLr family that are present on distal axons. These observations support the hypothesis that glial cells interact actively with axons of neurons in the brain to modulate the rate of axon extension.

Compartmented cultures of CNS neurons

Although compartmented cultures of sympathetic neurons from rats (56) and mice (57) have previously been established, attempts to culture CNS neurons in compartmented dishes have met with limited success. In one instance, hippocampal neurons in modified compartmented culture dishes produced short neurites, as is characteristic of these neurons (58). The ability to culture CNS neurons (RGC) that produce abundant axon growth in compartmented culture dishes is an important advance and offers several advantages over presently available systems for culturing CNS neurons. First, RGC are primary, post-mitotic neurons and the cultures are essentially devoid of other
cell types (32). These neurons extend long axons, a characteristic that facilitates their growth in compartmented dishes. Axons extend from the center, cell body-containing compartment into the side compartments at a rate of approximately 0.5 mm/day. The two side compartments contain pure distal axons, without cell bodies and dendrites (Fig. 1). Distal axons can be removed and the rate of axon regeneration can be accurately measured. Another important feature is that cell bodies and distal axons can be exposed to different milieux so that metabolic events occurring in distal axons can be modulated independently of those in cell bodies. Pure distal axons can also be harvested for biochemical measurements such as immunoblotting, enzymatic assays and radiolabeling. In addition, the compartmented culture system provides an excellent opportunity for studying mechanisms of transport of molecules between cell bodies and distal axons (40,59). This novel compartment model for culture of CNS neurons will be widely applicable to studies on the regulation of axon growth and regeneration of CNS neurons, and on the anterograde and retrograde transport of molecules between cell bodies and axons.

Glial cell-derived lipoproteins stimulate axon growth of retinal ganglion cells

Essentially all cholesterol in the brain is synthesized endogenously rather than being imported from the circulation (60). An abundant supply of cholesterol to neurons is important for a normal rate of axon growth. Previous studies have shown that when cholesterol synthesis was inhibited in cell bodies of sympathetic neurons from rats (43,46) and mice (40) the rate of axon extension was decreased. However, a normal
rate of axon growth was restored when cholesterol or human (low density and high density) lipoproteins were provided to distal axons (46).

It is not clear, however, what proportion of the cholesterol required for neuronal functions such as axon growth is derived from endogenous synthesis in neurons and what proportion is imported from surrounding glial cells. Although phospholipids can be synthesized in distal axons, cholesterol synthesis appears to be restricted to cell bodies (38,39). Consequently, cholesterol must be either anterogradely transported long distances from cell bodies to distal axons or, alternatively, imported from glial cells positioned in close proximity to the distal axons. Enigmatically, in response to a nerve injury, cholesterol synthesis in neurons is inhibited (61). At the same time, however, apo E synthesis in glial cells is dramatically increased (62,63). This increase in apo E production by glial cells has been proposed to represent a mechanism whereby lipid transport from glial cells to neurons is increased so that cholesterol is available for repairing the injured neurons (19,45,64). Pfrieger and colleagues have suggested that cholesterol synthesis in CNS neurons is down-regulated after birth, implying that under this condition most neuronal cholesterol would have to be imported from glial cells (65).

**Neuronal receptors of the LDLr family**

Neurons express multiple receptors of the LDLr superfamily, for example, the LDLr itself (45,46), the LDLr-related protein (30,47-49), the apo E receptor 2 (also known as LR7/8B) (46,50-52), the very low density lipoprotein receptor (53) and the LR11 receptor (also known as SorLA) (46,54,55). The LDLr (46), the LDLr-related protein (66,67) and the apo E receptor 2 (46) are expressed in axons. Based on the known
ligand specificity and function of the LDLr it is generally assumed that other members of this family of receptors can also bind and internalize apo E-containing lipoproteins via endocytosis. Consequently, cholesterol associated with LP-E derived from glial cells could be endocytosed by axons and used for membrane production during axon growth. Our observations are consistent with the idea that cholesterol delivered to distal axons by glial cell-derived apo E is an important source of cholesterol for normal axon growth of CNS neurons in vivo.

There are, however, other possible mechanisms by which glial cell-derived LP-E might stimulate axon growth since some receptors of the LDLr superfamily also act as signaling receptors upon binding to their ligands, such as apo E (53,68-70). Thus, important signaling pathways are activated in neurons by apo E-containing lipoproteins in the absence of internalization of the lipoprotein ligand. Importantly, in this mode of action, the receptor does not deliver lipids to the neurons. Some evidence that apo E can influence axon growth without delivering lipids came from experiments in which lipid-poor lipoproteins containing apo E3 or apo E4, but essentially no cholesterol, elicited differential effects on neurite growth of Neuro2a cells (31,42). In the present study, apo E alone, devoid of associated lipid, failed to stimulate axon extension of RGC. However, we are unable to conclude from this observation that cholesterol delivery from LP-E is required for stimulation of axon growth because lipid-free apo E appears not to be in the correct conformation for binding efficiently to the LDLr (24) or probably to other members of this receptor family. Consequently, our studies do not distinguish whether the growth stimulatory effect of glial LP-E is the result of lipid delivery to the axon or is due to activation of a signaling pathway that stimulates axon
growth. However, since RAP abrogates the growth stimulatory effect of glia-derived lipoproteins on RGC, we conclude that apo E-containing lipoproteins stimulate growth by binding to receptors of the LDLr family. An exciting possibility is that the compartmented culture technique will, in the future, be applicable to CNS neurons (e.g. RGC) derived from genetically-modified mice (e.g. those that do not express specific receptors of the LDLr family). Studies using these neurons would likely provide further mechanistic insight into the role of glial cell-derived lipoproteins in axon extension.

Our data add to the growing body of evidence suggesting that glial cells have an intimate relationship with neurons and are important for normal neuronal functions such as neuronal excitability, synaptic transmission and synapse formation (2-7,10). Interestingly, cholesterol in GCM promotes synapse formation in RGC, in a process most likely mediated by apo E (10). In support of this concept, a recent study showed that cholesterol- and sphingolipid-rich lipid rafts are abundant in dendrites of cultured hippocampal neurons and that depletion of cholesterol and rafts leads to gradual loss of synapses and dendritic spines (71). The present study demonstrates for the first time that apo E-containing lipoproteins secreted from glial cells stimulate axon growth of CNS neurons when applied locally to distal axons.
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1Abbreviations used: apo, apolipoprotein; BM, base medium; CNS, central nervous system; GCM, glial cell-conditioned medium; LDLr, low density lipoprotein receptor; LP-E, apo E-containing lipoproteins; RAP, receptor-associated protein; RGC, retinal ganglion cells.
REFERENCES


FIGURE LEGENDS

Figure 1. **Compartmented cultures of retinal ganglion cells (RGC).** RGC from one-day-old rats are plated in the center compartment of 3-compartmented culture dishes and axons extend along tracks into left and right compartments. Top panel: representation of cell bodies/proximal axons in the center compartment, the Teflon divider, and distal axons growing along one track (bounded by 2 horizontal scratches) into the right-hand compartment. Bottom panel: RGC were stained with anti-tau-1 antibody (red, marker of axons) and anti-MAP-2 antibody (green, marker of dendrites) showing that side compartments contain distal axons alone whereas cell bodies stain with both antibodies. Size bar represents 100 µm.

Figure 2. **Glial cell-conditioned medium (GCM) from rat cortical glial cells promotes axon extension.** Retinal ganglion cells were cultured in compartmented dishes for 7 to 10 days then distal axons in side compartments were axotomized. (A) The following media were added to regenerating axons in side compartments: base medium with no additions (BM, open circles); base medium with growth factors (brain-derived neurotrophic factor and ciliary-derived neurotrophic factor) (GF, filled triangles); GCM (filled circles). Axon extension was measured each day for 4 days. Data are means ± S.E. (n = 90) of 3 dishes with 30 tracks measured/dish. The experiment is representative of 5 similar experiments. * indicates statistically different from BM on day 4 (p < 0.002). (B) Base medium with no additions (BM, open circles), base medium containing rat serum (1 µg cholesterol/ml) (filled triangles) or base medium containing
fetal bovine serum (1 μg cholesterol/ml) (open triangles) was added to distal axons and axon extension was measured. Data are means ± S.E. (n = 90) of 3 dishes with 30 tracks measured/dish. The experiment was repeated with similar results.

Figure 3. **Compactin inhibits the secretion of apo E and cholesterol from glial cells.** (A) Glial cells were incubated with the indicated concentrations of compactin for 3 days, then the glial cell-conditioned medium (GCM) was removed and subjected to polyacrylamide gel electrophoresis and immunoblotting with anti-apo E and anti-apo J antibodies. The experiment was repeated twice with similar results. (B) The cholesterol content of GCM from compactin-treated glial cells was measured by gas-liquid chromatography. Data are means ± S.E. of 3 independent experiments. * and ** indicate statistically different ($p < 0.0005$ and $p < 0.0001$, respectively) from samples incubated without compactin. (C) Base medium (BM, open circles) or GCM from glial cells incubated for 3 days with 0 (GCM, filled circles), 100 (open triangles), 300 (closed triangles) or 1,000 (filled squares) nM compactin, was added to side compartments of axotomized RGC for the indicated times and axon extension was measured. Data are means ± S.E. (n = 60) of 30 tracks measured/dish in duplicate dishes. The experiment was repeated with similar results. Statistical significance from BM after 4 days is indicated by * $p < 0.02$ and ** $p < 0.001$.

Figure 4. **Cholesterol or apo E alone do not enhance axon extension.**

Compartmented cultures of retinal ganglion cells were axotomized and human apo E3 (1 or 10 μg/ml from PanVera, Madison, WI) or cholesterol (1 or 10 μg/ml in ethanolic
solution) was added to distal axons in base medium. Axon extension was measured after 4 days. Data are means ± S.E. (n = 90) of 30 tracks/dish from triplicate dishes.

Figure 5. **Isolation of apo E-containing lipoproteins from glial cell-conditioned medium.** Glial cell conditioned medium (GCM) was subjected to sucrose density gradient ultracentrifugation. Fractions with densities 1.04 to 1.27 g/ml were immunoblotted with anti-apo E antibody (upper panel). Apo E was quantitated by densitometric scanning of the immunoblot (lower panel). Apo E-containing fractions (typically fractions 5 to 8) were combined and designated LP-E. Densities of fractions (g/ml) are indicated by the dashed line.

Figure 6. **Apo E-containing lipoproteins (LP-E) promote axon growth of retinal ganglion cells (RGC).** (A) RGC were axotomized and base medium (BM, open circles), GCM (cholesterol content = 1 µg/ml; filled circles) or base medium with LP-E (LP-E, final cholesterol concentration = 1 µg/ml; filled triangles) was added to the side compartments. (B) The center compartment alone, containing cell bodies with proximal axons, was incubated in base medium containing B-27 and growth factors without (- LP-E) or with (+ LP-E) glial cell lipoproteins (1 µg/ml cholesterol). Axon extension was measured. In panels a and b data are means ± S.E. (n = 90) of 30 tracks/dish in triplicate dishes. The experiments were repeated 3 times with similar results. * and ** indicate statistically different (p < 0.0001 and p < 0.002, respectively) compared to BM after 4 days.
Figure 7. **Glial cell-derived apo E-containing lipoproteins (LP-E) do not enhance axon extension of rat sympathetic neurons.** Medium containing (+ LPE, filled circles) or lacking (- LP-E, open circles) LP-E (1 µg cholesterol/ml) was added to distal axons of compartmented cultures of rat sympathetic neurons. Axon extension was measured. Data are means ± S.E. (n = 90) of 30 tracks measured/dish in triplicate dishes. The experiment was repeated twice with similar results.

Figure 8. **Apo E-containing lipoproteins (LP-E) restore the ability of medium from compactin-treated glial cells to promote axon growth.** (A) Base medium (BM, open circles), GCM (filled circles) or GCM from glial cells that had been incubated with 1,000 nM compactin and containing (+ LP-E, filled squares) or lacking (- LP-E, open squares) LP-E (1 µg cholesterol/ml), was provided to distal axons. Axon extension is shown as the mean ± S.E. (n = 90) of 30 tracks/dish from triplicate dishes. * and ** indicate statistically different (p < 0.003 and p < 0.0001, respectively) compared to BM after 4 days. (B) GCM was depleted of lipoproteins by incubation with Cab-o-sil. Cab-o-sil-treated GCM (indicated as Cab) was immunoblotted with anti-apo E antibodies and anti-apo J antibodies. (C) Distal axons of RGC were incubated with lipoprotein-depleted GCM (Cab, filled triangles), GCM (filled circles) or base medium (BM; open circles) and axon extension was measured. Axon extension is shown as mean ± S.E. (n = 90) of 30 tracks/dish from triplicate dishes. * indicates statistically different from BM and Cab (p < 0.0003 and p < 0.0019, respectively) after 4 days. The Bonferroni statistical analysis was used.
Figure 9. **Receptor-associated protein (RAP) abrogates the growth stimulatory effect of glial cell-conditioned medium (GCM).** Distal axons of RGC were incubated with GCM (filled circles), base medium (BM; open circles), GCM + 300 nM RAP (G+R; filled triangles) or BM + RAP (B+R; open triangles). Axon extension was measured and is given as mean ± S.E. (n = 90) of 30 tracks measured/dish from triplicate dishes. * indicates statistically different from BM, G+R and B+R (p < 0.0011, p < 0.0235 and p < 0.0043, respectively) after 4 days. The Bonferroni statistical analysis was used.
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A

B

Axonal extension (mm)

Time after axotomy (day)

Axonal extension (mm)

Time after axotomy (day)
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Figure 4  H. Hayashi et al.
Figure 5  H. Hayashi et al.
Figure 6  H. Hayashi et al.
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Hideki Hayashi, Robert B. Campenot, Dennis E. Vance and Jean E. Vance

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