Uptake of Lipoproteins for Axonal Growth of Sympathetic Neurons*  

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Lipoproteins originating from axon and myelin breakdown in injured peripheral nerves are believed to supply cholesterol to regenerating axons. We have used compartmented cultures of rat sympathetic neurons to investigate the utilization of lipids from lipoproteins for axon elongation. Lipids and proteins from human low density lipoproteins (LDL) and high density lipoproteins (HDL) were taken up by distal axons and transported to cell bodies, whereas cell bodies/proximal axons internalized these components from only LDL, not HDL. Consistent with these observations, the impairment of axonal growth, induced by inhibition of cholesterol synthesis, was reversed when LDL or HDL were added to distal axons or when LDL, but not HDL, were added to cell bodies. LDL receptors (LDLRs) and LR7/8B (apoER2) were present in cell bodies/proximal axons and distal axons, with LDLRs being more abundant in the former. Inhibition of cholesterol biosynthesis increased LDLR expression in cell bodies/proximal axons but not distal axons. LR11 (SRoLA) was restricted to cell bodies/proximal axons and was undetectable in distal axons. Neither the LDL receptor-related protein nor the HDL receptor, SR-B1, was detected in sympathetic neurons. These studies demonstrate for the first time that lipids are taken up from lipoproteins by sympathetic neurons for use in axonal regeneration.

Axonal elongation requires the expansion of axonal membranes by addition of new membrane materials (proteins and lipids) to the growing axon. In sheep (1) and rats (2, 3) the brain and peripheral nerves synthesize all the cholesterol needed for development without requiring cholesterol from circulating lipoproteins. Moreover, after birth, cholesterol used for myelin production is made locally (4). In contrast, fetal liver supplies about 50% of the cholesterol needed for development of heart, lung, and kidney (3). Surprisingly, during peripheral nerve regeneration, cholesterol synthesis in the nerve is down-regulated (5), yet serum-derived cholesterol does not contribute significantly to myelin synthesis or axonal regeneration (6, 7).

Instead, cholesterol from degenerating axons and myelin is proposed to be retained within the nerve and re-utilized via a lipoprotein-mediated process (8), although cholesterol uptake by neurons was not directly demonstrated. The presence of endoneurial lipoproteins in regenerating, but not non-injured, nerves is well documented. These lipoproteins contain apolipoprotein (apo) E and apoAI but not apoB (9–11). After peripheral nerve injury, apoE synthesis by resident macrophages increases greatly, and apoE accumulates within the nerve (12–14) supporting the concept that apoE is involved in re-utilization of lipids from degenerating nerves for axonal regeneration and myelin production.

The receptors involved in lipoprotein uptake by axons and Schwann cells have not been fully characterized, nor has the uptake and utilization of lipids from lipoproteins for axonal regeneration been directly demonstrated. The low density lipoprotein receptor (LDLR) has been reported to be used by PC12 cells, Schwann cells, and sensory neurons in vitro for uptake of plasma lipoproteins and endoneurial lipoproteins isolated from crushed sciatic nerves (15, 16). In vivo, the tips of regenerating axons contain a high concentration of LDLRs (9), and LDLRs are distributed throughout all regions of PC12 cells (15). Thus, the model originally proposed for re-utilization or “salvage” of cholesterol during nerve regeneration involved apoE-containing lipoproteins and LDLRs.

More recently, other lipoprotein receptors have been detected in neurons. In adult rats, the LDLR-related protein (LRP), a multifunctional apoE receptor, was found to be highly expressed in neurons of brain and spinal cord (17, 18) and was shown to modulate hippocampal neurite development (19). LRP was also detected in neuronal cell bodies and proximal processes in adult human brain (20, 21). In cultured hippocampal neurons, LRP is restricted to the somatodendritic domain (22). Two other receptors of the LDLR family that are predominantly expressed in the brain, LR11 (SorLA) (23–25) and LR7/8B (apoER2) (26–28), have been identified. Since LR11 and LR7/8B are highly expressed in neurons, and LR11 expression is regulated during central nervous system (CNS) development (24), these receptors have been suggested to be involved in neural organization, synaptic formation, and neurodegenerative diseases such as Alzheimer’s disease (29).

The lipoprotein receptors present in neurons of the peripheral nervous system (PNS) have not yet been rigorously examined although both the LDLR and LRP have been detected in rabbit dorsal root ganglia neurons (30). A reasonable prediction is that a different spectrum of lipoprotein receptors might be present in CNS and PNS neurons since the classes of lipopro-

1 The abbreviations used are: apo, apolipoprotein; CNS, central nervous system; DI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDL, high density lipoproteins; LDL, low density lipoproteins; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; PNS, peripheral nervous system; SR-B, scavenger receptor class B; h, human; c, chicken.
teins that these two types of neurons would encounter are different. For example, in the PNS, distal axons would be exposed to the same types of lipoproteins as in the circulation (i.e. very low density lipoproteins, LDL, and HDL). In contrast, apoB-containing lipoproteins (LDL and very low density lipoproteins) appear to be absent from cerebrospinal fluid. Instead, lipoproteins in the vicinity of CNS neurons are HDL-sized and contain apoE, apoAI, apoD, and/or apoJ (31–34).

We have previously shown that cholesterol synthesis is restricted to cell bodies/proximal axons of sympathetic neurons and is not detectable in distal axons (35). When cholesterol synthesis was inhibited by pravastatin, axonal growth was severely impaired. However, normal growth was restored when cholesterol was added to either cell bodies or distal axons (36). Serum lipoproteins also restored normal axonal growth to these neurons. When LDL were given to either cell bodies or axons of pravastatin-treated neurons, axonal elongation proceeded normally, presumably because the LDL supplied cholesterol. In contrast, HDL restored axonal growth when added only to distal axons but not to cell bodies. These observations suggest that sympathetic neurons express multiple lipoprotein receptors and that these receptors have a polarized distribution.

We now show that lipids can be supplied to sympathetic neurons from lipoproteins for use in axonal growth. Our data are consistent with the model that the uptake of lipoprotein components by these neurons occurs via receptor-mediated endocytosis. We show that the LDLR is present in both cell bodies and axons of rat sympathetic neurons, and we have identified two additional members of the LDL receptor superfamily, LR7/8B (apoER2) and LR11 (SorLA), in these neurons. However, neither LRP (which is present in CNS neurons) nor the HDL receptor, SR-BI, was detected. In addition, the experiments show that lipoprotein receptors are differentially distributed and regulated in cell bodies and distal axons of sympathetic neurons.

EXPERIMENTAL PROCEDURES

Materials—[1α,2α-3H]Cholesterol (specific activity 45 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Pravastatin was a gift from Dr. S. Yokoyama, Nagoya City University Medical School, Japan. 1,1'-Diiodoctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and the Alexa 488 protein labeling kit were from Molecular Probes, Inc. (Eugene, OR). L15 medium without antibiotics was supplied by Life Technologies, Inc. Mouse 2.5 S nerve growth factor was from Alomone Laboratories Ltd. (Jerusalem, Israel). Rat serum was provided by the University of Alberta Laboratory Animal Services. The anti-rat LDLR polyclonal antibody was a gift from Dr. G. Ness, University of South Florida (37). A polyclonal antibody directed against the carboxy-terminal 14 amino acids of the chicken LR7/8B (apoER2) receptor (26) was generously provided by Dr. J. Nimpf, University of Vienna, Austria. The anti-murine SorLA (LR11) polyclonal antibody, raised against a fusion protein containing the fibronectin domain of SorLA, was a gift from Dr. H. Schaller, University of Hamburg, Germany (23). The anti-SR-BI antibody, directed against a peptide corresponding to amino acids 495–509 from murine SR-BI was kindly provided by Dr. M. Krieger, Massachusetts Institute of Technology (38). The rabbit anti-LRP polyclonal antibody was generated against the 85-kDa fragment of rat LRP and was a gift from Dr. J. Herz, University of Texas Southwestern Medical Center (39). Electrophoresis reagents were supplied by Bio-Rad. Polyvinylidene difluoride membranes were from Millipore. All other reagents were from Sigma or Fisher.

Preparation of Neuronal Cultures—Procedures for growing rat sympathetic neurons in compartmented cultures have been previously reported (40). Briefly, superior cervical ganglia from newborn Harlan Sprague-Dawley rats were dissected and enzymatically and mechanically dissociated. The cells were plated in either the center or the left compartment of three-compartmented culture dishes (36). Neurons were cultured for 10–14 days prior to the start of experiments. For measurements of axonal extension, left-plated cultures were used; all other experiments were performed using center-plated cultures.

Lipoprotein Isolation and Labeling—Human LDL (hLDL) and human HDL, (hHDL), were isolated from plasma by sequential ultracentrifugation and affinity chromatography (36). Lipoprotein preparations were monitored by SDS-polyacrylamide gel electrophoresis to confirm the expected apoprotein compositions and to ensure that hLDL did not contain apoE. Chicken HDL (cHDL) was isolated by sequential ultracentrifugation as the fraction of density between 1.12 and 1.21 g/ml (41).

Lipoproteins were labeled with DiI as described previously (42). Briefly, 4 mg of hLDL, hHDL, or cHDL were added to 8 ml of lipoprotein-deficient cell serum. DiI diethyl sulfoxide (200 μl, 3 mg/ml) was added, and the mixture was incubated at 37 °C for 18 h. Subsequently, the density was adjusted to 1.063 g/ml for hLDL and 1.21 g/ml for hHDL, and cHDL, by addition of KBr. Lipoproteins were re-isolated at 4 °C by centrifugation at 150,000 × g for 24 h in a Beckman Ti50.2 rotor. The top 2 ml containing DiI-labeled lipoproteins, were collected by aspiration and dialyzed against 0.9% NaCl and 0.01% EDTA. Subsequently, Dil-lipoproteins were filtered through 0.45-μm filters, stored under sterile conditions at 4 °C, and used within a month. Dil-lipoproteins were subjected to SDS-polyacrylamide gel electrophoresis on 3–15% gradient gels. Gels were analyzed visually for DiI fluorescence and Coomassie Blue staining for identification of major apoproteins. In hHDL, bands corresponding to apoAI and apoAII were not labeled with DiI; the same was true for apoAI in cHDL. For hLDL, most fluorescence was at the gel front as free DiI. However, some Dil fluorescence was associated with apoB100 since some lipids remain associated with apoB under these conditions.

Alexa 488-labeled lipoproteins were prepared according to manufacturer’s instructions. Alexa Fluor 488 dye reacts with primary amines of proteins forming stable dye-protein conjugates. In HDL, Alexa 488 binds covalently to apoAI and apoAII (43). SDS-polyacrylamide gel electrophoresis confirmed that apoB, apoAI, apoAII, and chicken apoAI were labeled. All lipoprotein preparations were essentially free of unbound Alexa. Recovery of Alexa fluorescence in the aqueous phase after extraction of Alexa lipoproteins with organic solvents was greater than 95% providing evidence that the label was primarily associated with proteins.

Lipoproteins were labeled with [1α,2α-3H]cholesterol as described elsewhere (44). Briefly, the solvent from 200 μCi of [1α,2α-3H]cholesterol was evaporated to form a thin film. The lipoprotein preparation (3 μg of protein) was added in 0.9% NaCl, and the mixture was shaken overnight at 4 °C, after which lipoproteins were re-isolated by flotation as described above. The specific radioactivity of cholesterol (dpm/μg total cholesterol) was determined. Cholesterol mass was determined by an enzymatic kit (Sigma). Typically, the specific radioactivity of cholesterol was 2900 dpm/μg for hLDL, 150,200 dpm/μg for hHDL, and 64,100 dpm/μg for cHDL.

Analysis of Lipoprotein Uptake by Fluorescence Microscopy—Neurons were plated in the center compartment of compartmented dishes and cultured for 14 days. Dil or Alexa lipoproteins were added to the cell body- or distal axon-containing compartments as indicated. Lipoprotein concentration in media was normalized to 100 μg of total cholesterol/ml. In addition, fluorescence units were normalized to achieve the same fluorescence in all media by addition of unlabeled lipoproteins. After 18 h, the neurons were washed extensively with cold phosphate-buffered saline containing methylocellulose (3 g/500 ml) and fixed for 20 min in 4% paraformaldehyde. Coverslips were secured using para-phenylenediamine in glycerol as mounting medium. Fluorescence microscopy was performed with an Olympus BX-50F stereomicroscope equipped with a 100-watt gas mercury lamp and rhodamine filter (for Dil). Alternatively, neurons were analyzed by confocal laser scanning microscopy using a Leica microscope illuminated by a 100-watt mercury burner for direct observation, a Ar/Kr laser with major emissions at 488, 568 and 647 nm for scanning, and 63 × 1.40 NA oil immersion objectives. Figs. 2, 4, and 5 were prepared using Adobe Photoshop software.

Association of DiI and [1α,2α-3H]Cholesterol with Neurons—Neurons were incubated with Dil lipoproteins in the cell body-containing compartment for 18 h and then washed three times with ice-cold phosphate-buffered saline. Cellular material was harvested from the center compartment in phosphate-buffered saline. The detached neurons were collected by centrifugation for 10 s with a Beckman TLA-100. An aliquot was added to 2 ml of methanol, and fluorescence was analyzed in a Hitachi F-2000 spectrofluorometer with excitation light of 550 nm and emission at 565 nm. Protein content was determined using the BCA protein kit (Pierce). Dil did not interfere with the protein determinations. In experiments with
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[^3H]Cholesterol-labeled lipoproteins, neurons were given labeled lipoproteins in the cell body-containing compartment. The concentration of lipoproteins was adjusted so that the cholesterol content was 100 μg/ml, and the same specific radioactivity of cholesterol was achieved for all lipoproteins by addition of unlabeled lipoproteins. After 18 h, the cells were washed, and material from the center compartment was harvested, and radioactivity was measured. The amount of cell-associated lipoprotein-derived cholesterol was calculated from the specific radioactivity of the lipoprotein[^3H]cholesterol.

**Measurement of Axonal Extension**—Neurons were plated in the left compartment of compartmented dishes. Distal axons were mechanically removed from the right-hand compartment with a jet of sterile distilled water delivered with a syringe through a 22-gauge needle. The water was aspirated and the wash repeated twice, after which fresh medium was added. This procedure, termed axotomy, effectively removes all visible traces of axons from the right-side compartment. Axonal growth was measured as described previously (45).

**Analysis of Lipoprotein Receptors by Immunoblotting**—Material from distal axon- and cell body-containing compartments was harvested separately in 50 mM Tris-Cl (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 0.05 mM phenylmethylsulfon fluoride. Cellular material was sonicated for 10 s with a probe sonicator and centrifuged at 350,000 × g for 20 min. Membrane pellets were dissolved in 50 mM Tris-Cl (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 0.05 mM phenylmethylsulfonyl fluoride. The protein content was determined using the BCA protein kit (Pierce). Proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel for LDLR and SRBI, a 5% gel for LR11 and LR7/8B, and a 3–15% gradient gel for LRP. For LR7/8B, electrophoresis was performed under non-reducing conditions (28). Proteins were transferred to polyvinylidene difluoride membranes for 18 h at 25 V (36). Membranes were incubated in 20 mM Tris-Cl (pH 7.5), 500 mM NaCl, 0.1% (v/v) Tween 20 (TTBS) with 5–10% (w/v) non-fat dried milk. Membranes were then incubated for 1 h at room temperature with the following dilutions of primary antibodies: LDLR, 1:1000; LR11, 1:1500; SR-BI, 1:1000; LR7/8B, 1:15000; and LRP, 1:5000. Membranes were then incubated for 1 h with horseradish peroxidase linked to anti-rabbit IgG secondary antibody (Pierce, diluted 1:10,000), and immunoreactive proteins were detected by enhanced chemiluminescence.

**RESULTS**

**Lipoproteins and Axonal Growth**—We have previously shown that inhibition of cholesterol synthesis by pravastatin impairs axonal elongation of compartmented cultures of rat sympathetic neurons. Addition of cholesterol to axons or cell bodies restored normal axonal growth to pravastatin-treated neurons. Similarly, when human lipoproteins (hLDL, hHDL2, or hHDL3) were supplied to distal axons of pravastatin-treated neurons, normal axonal growth occurred. In contrast, normal axonal elongation occurred only when hLDL, but not hHDL3 or hHDL2, were added to the cell body-containing compartment (36). These studies suggested that cholesterol can be taken up from lipoproteins by these neurons and used for axonal growth. The observations also raised the possibility that LDL and HDL, or the cholesterol therein, are taken up by different mechanisms, potentially involving distinct lipoprotein receptors. We have now extended these studies and analyzed the capacity of hLDL, hHDL3, and chicken HDL (cHDL) to deliver lipids, including cholesterol, for axonal growth of rat sympathetic neurons. hHDL3 lacks apoE and, therefore, would not be expected to interact with the apoE receptor (i.e., the LDLR). cHDL was selected because chicken apoAI has been proposed to play a role in nerve regeneration in chickens similar to that of apoE in mammals (46–48). Consequently, we expected cHDL and hLDL to be similarly taken up by rat sympathetic neurons. For these experiments, rat sympathetic neurons were plated in the left side compartment of three-compartmented culture dishes. In these cultures (49), all compartments contain independent fluid environments, and the cell bodies/proximal axons reside in a compartment completely separate from that containing distal axons. After 14 days, axons in the right side compartment were axotomized, and cholesterol synthesis was inhibited by addition of 50 μM pravastatin to the cell body-containing compartment. This concentration of pravastatin inhibits the incorporation of [14C]acetate into cholesterol by ~80% (50). The concentration of each lipoprotein preparation added to cell bodies was equalized in terms of cholesterol content (100 μg/ml). Axonal extension was measured after 4 days (Fig. 1). In agreement with our previous results (36), axonal extension was reduced by ~50% in pravastatin-treated cells compared with untreated cells, whereas the addition of hLDL, but not hHDL3, to cell bodies/proximal axons restored axonal elongation. In addition, Fig. 1 shows that axons extended normally when cHDL were added to cell bodies/proximal axons of pravastatin-treated neurons. These data suggest that hHDL2 cannot provide cholesterol to cell bodies for axonal growth, possibly because cell bodies lack a receptor for hHDL2. In accordance with our previous observations (36), when any of the lipoproteins was given to distal axons alone, the axons extended normally (data not shown).

**Uptake of Lipids and Proteins from Lipoproteins**—We investigated whether or not lipids and proteins from lipoproteins were taken up by cell bodies of sympathetic neurons. hLDL, cHDL, and hHDL3 were labeled with DiI, a hydrophobic fluorescent dye that intercalates into the neutral lipid core of lipoproteins without affecting receptor binding (51). Rat sympathetic neurons were maintained for 14 days and then incubated with DiI lipoproteins in the center compartment containing the cell bodies. The concentration of lipoproteins in the media was adjusted so that the total cholesterol concentration (100 μg/ml medium) and specific fluorescence (fluorescence intensity units/ml of medium) were the same for all lipoproteins. After 18 h, the neurons were extensively washed and examined by fluorescence microscopy. Fig. 2, a, c, and e, shows the bright field images of cell bodies/proximal axons of neurons given hLDL, cHDL, and hHDL3, respectively. Fig. 2, b, d, and f, shows the corresponding fluorescence images. Cell bodies of neurons given DiI-hLDL or DiI-cHDL exhibited intense fluorescence (Figs. 2, b and d), whereas almost no fluorescence was visible in cell bodies of neurons given hHDL3 (Fig. 2f). For quantification of the fluorescence associated with cell bodies, cellular material was harvested from the center compartment, and DiI fluorescence intensity was measured. Neurons treated with hLDL and cHDL contained 4- and 3-fold, respectively, more fluorescence than did neurons given hHDL3 (Fig. 3a). Assuming that DiI is a valid marker for uptake of neutral lipids from lipoproteins (52), these data suggest that cell bodies/
proximal axons internalize lipids from hLDL and chDL but not hHDL3.

We next measured the uptake of [3H]cholesterol from lipoproteins by cell bodies. Neurons were plated in the center compartment and maintained for 14 days, after which cell bodies/proximal axons were incubated with [3H]cholesterol-labeled lipoproteins for 18 h. The cells were then washed and harvested, and radioactivity was determined. Neurons incubated with hLDL and chDL contained approximately 70% more radioactivity than did neurons incubated with hHDL3 (Fig. 3b). Although Fig. 3, a and b, indicates that the association of lipids from hLDL and chDL by cell bodies is greater than from hHDL3, relatively more [3H]cholesterol-labeled hHDL3 than Dil-labeled hHDL3 associated with the neurons. One likely explanation for this difference is that in addition to hHDL3, specifically binding to the cell surface, [3H]cholesterol on the HDL surface might exchange with cell membranes via aqueous diffusion. In contrast, Dil is present in the neutral lipid core of HDL3 and would, therefore, not exchange via aqueous diffusion on to the cell surface but would associate with cells only via a specific receptor-mediated interaction.

The experiments described above suggest that lipids from hLDL and chDL, but not hHDL3, are taken up by the neuronal cell bodies. Since in our experience these neurons are particularly sensitive to cold temperatures, we were unable to perform classical lipoprotein binding experiments at 4°C to differentiate between uptake and nonspecific binding of lipoproteins. To exclude the possibility that the observations described above could be explained solely by the lipoproteins binding to the cell surface, without their internalization, we used confocal laser scanning microscopy to examine more directly fluorescent lipoprotein uptake. By using this technique we were also able to determine whether or not distal axons can take up lipids and proteins from lipoproteins and transport them to the cell bodies, since the only way in which components of lipoproteins that have been added to distal axons can enter the cell bodies is from retrograde, intracellular transport. Thus, the appearance of fluorescence in cell bodies, after addition of lipoproteins containing fluorescent lipids and proteins to distal axons, would unambiguously demonstrate that lipids and proteins, respectively, had been taken up from lipoproteins by distal axons and transported to cell bodies. For these experiments, lipoproteins were labeled with either DiI or Alexa 488 (a fluorescent label of proteins) and added to either distal axons or cell bodies/proximal axons. Lipoprotein concentrations were adjusted in terms of total cholesterol content (100 μg/ml medium) and specific fluorescence. The protein components of the lipoproteins did not bind significant amounts of DiI, and conversely, Alexa 488 associated almost exclusively with proteins of the lipoproteins (see “Experimental Procedures”). After 18 h, cell bodies were examined for fluorescence. All images were analyzed on a plane where nuclei were visible to ensure that the fluorescent lipoprotein did not only adhere to the cell surface but was truly intracellular.

When cell bodies/proximal axons were incubated with fluorescent hLDL or chDL, intense Dil (Fig. 4, B and D) and Alexa (Fig. 4, H and J) fluorescence, likely representing aggregates of lipoproteins, was observed inside the cell bodies. In contrast, when either DiI- or Alexa-hHDL3 was given to the cell body-containing compartment, little fluorescence was visible in cell bodies (Fig. 4, F and L). The ring of fluorescence in Fig. 4L probably represents Alexa lipoproteins remaining on the cell surface after the extensive washing procedure. Even when the experiments were repeated using a 10-fold higher concentration of DiI- or Alexa-hHDL3, the fluorescence inside the cell bodies remained at basal levels. These experiments show that lipids and proteins from hLDL and chDL, but not hHDL3, are internalized by cell bodies/proximal axons. In parallel experiments, neurons that were given DiI-hLDL (Fig. 4A), DiI-chDL (Fig. 4C), or DiI-hHDL3 (Fig. 4E) to distal axons exhibited profuse punctate fluorescence in the cell bodies, suggesting that lipids from all the lipoproteins were taken up by distal axons and transported to cell bodies. These results are in accordance with our observations that all these lipoproteins, given to distal axons, can overcome the inhibition of axonal growth induced by pravastatin (36). The uptake of Alexa-labeled hLDL, chDL, and hHDL3 by distal axons was similarly examined. Fig. 4, G, I, and K, shows that each of the Alexa lipoproteins was taken up by distal axons and transported to cell bodies. These data demonstrate that lipids and proteins from hLDL, chDL, and hHDL3 can be internalized by distal axons and transported to cell bodies. In addition, lipids and proteins from hLDL and chDL, but not hHDL3, were internalized by cell bodies/proximal axons. Our findings also confirm that the various lipoprotein classes are differentially taken up by cell bodies and distal axons of sympathetic neurons. Previous studies have reported that in several cell types a selective uptake of lipids, particularly cholesteryl esters, occurs from HDL (43), whereas the uptake of HDL holoparticles has been less well documented (53, 54). Therefore, our observation that distal axons of sympathetic neurons were able to internalize both lipids and proteins from hHDL3 was somewhat unexpected.

We next investigated whether or not uptake of hLDL and hHDL3 occurred via receptor-mediated processes. Neurons were given DiI- or Alexa-labeled lipoproteins to either distal axons or cell bodies/proximal axons. Some cultures were given a 50-fold excess of unlabeled lipoproteins for competition of uptake. Unlabeled hLDL competed effectively for uptake of fluorescent hLDL by cell bodies (compare Fig. 5, A with D, and
**Fig. 3.** Quantitation of fluorescent and radiolabeled lipids associated with cell bodies. a, neurons were incubated with Dil-lipoproteins as in Fig. 2 legend. After extensive washing, material from the center compartment was harvested and Dil fluorescence measured. b, neurons cultured as in Fig. 2 were incubated with [3H]cholesterol-lipoproteins for 18 h, then washed, harvested and the amount of cell-associated, lipoprotein-derived cholesterol was calculated from the specific radioactivity of [3H]cholesterol in the lipoprotein. Values are means ± S.E. of six determinations for each treatment. Statistically significant differences compared with treatment with hLDL are indicated by * and were evaluated by the Student’s t test (p < 0.05). The experiment was repeated twice with similar results.

**FIG. 4.** Uptake of fluorescent lipids and proteins from lipoproteins. Center-plated neurons were incubated with Dil- or Alexa-lipoproteins in either the cell body-containing compartment (B, D, F, H, J, and L) or distal axon-containing compartment (A, C, E, G, I, and K). Lipoprotein concentration was adjusted to 100 μg of cholesterol/ml and 136,000 fluorescence intensity units/ml for Dil lipoproteins or 180,000 fluorescence intensity units/ml for Alexa lipoproteins. After 18 h, neurons were processed for confocal microscopy. The images shown for each group (DiI and Alexa) were captured under identical conditions, and 50–100 fields were analyzed for each treatment. The experiment was repeated twice with comparable results.

**Fig. 5.** Competition of fluorescent lipoprotein uptake by unlabeled lipoproteins. Neurons were incubated in either the cell body-containing (A, D, G, and J) or distal axon-containing (B, C, E, F, H, I, K, and L) compartment with fluorescent lipoproteins, as in Fig. 4 legend. Some cultures were given a 50-fold excess of unlabeled lipoproteins. Cell bodies were visualized by confocal microscopy, and the images shown for each group (DiI and Alexa) were captured under identical conditions. The experiment was repeated twice with similar results.

**Lipoprotein Receptors in Sympathetic Neurons—**The distribution of lipoprotein receptors in cell bodies and distal axons of sympathetic neurons was investigated by immunoblot analysis. Neurons were cultured both in 24-well dishes (designated “mass cultures”) and compartmented dishes. Cellular material was harvested, a membrane-enriched fraction was prepared, and membrane proteins were separated by SDS-polyacrylamide gel electrophoresis, after which immunoblotting was performed. Proteins from homogenates of rat liver and rat brain were used as controls. Fig. 6a shows that the LDLR (Mr ~130) is widely distributed throughout cell bodies and axons of sympathetic neurons but is more abundant in cell bodies/proximal axons than in distal axons. As a positive control, the
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LDLR was also detected in rat liver and rat brain. In contrast, the 85-kDa fragment of LRP was not detected in any preparation of sympathetic neurons. However, this receptor was present in rat liver and rat brain, which were used as positive controls. We were surprised that we were unable to detect LRP in sympathetic neurons since LRP has been reported to be abundant in CNS neurons (20) and brain (18, 21). We considered the possibility that our inability to detect LRP was due to a factor in the neuron samples that interfered with immunodetection of LRP. This possibility was eliminated when a rat brain homogenate was mixed with membranes from neuronal mass cultures (shown as Rat Brain* in Fig. 6a), and the intensity of the band representing brain LRP was not diminished. Fig. 6 also shows that the HDL receptor, SR-BI, is present in rat liver but not brain, in agreement with the distribution of SR-BI mRNA (38). No immunoreactive SR-BI was detected in sympathetic neurons.

In many cell types, expression of the LDLR is regulated by cellular cholesterol content (55). We therefore investigated whether or not inhibition of cholesterol biosynthesis increased expression of the LDLR in cell bodies/proximal axons and distal axons of sympathetic neurons. Compartmented neuron cultures were treated with 50 μM pravastatin (Prav) for 48 h. Cellular material was harvested, and the presence of the LDLR (LDLr) analyzed by immunoblotting. This experiment was performed four times with independent preparations of neurons. In addition, two different amounts of axonal proteins (20 and 60 μg) were applied to the gel. The same result was obtained for each experiment.

The presence of two other receptors of the LDL receptor superfamily that are abundant in brain, LR7/8B (also known as SorLA) (23, 25, 26, 56), was also examined by immunoblotting. Fig. 7 shows the presence of LR11 (Mf ~240) in rat brain and cell bodies/proximal axons but the absence of LR11 from distal axons and rat liver. LR7/8B (Mf ~130) was detected in both cell bodies/proximal axons and distal axons, as well as in rat brain but not in rat liver.

**DISCUSSION**

Our previous experiments indicated that lipoproteins given to distal axons of rat sympathetic neurons can provide cholesterol, but not phospholipids, for axonal regeneration (36). We now show for the first time that distal axons internalize proteins and lipids from lipoproteins and transport these components retrogradely to cell bodies. In contrast, cell bodies/proximal axons take up lipids and proteins from LDL but not HDL3. The results are consistent with the idea that both lipid and protein components of lipoproteins are taken up by axons via receptor-mediated endocytosis and transported to cell bodies, the site of lysosomal processing. However, the possibility exists that some components (e.g., cholesteryl esters and/or cholesterol) remain in axons and are directly used for growth.

The LDL Receptor in Sympathetic Neurons—Lipoprotein receptors are known to play an important role in cellular cholesterol homeostasis (55), and our experiments indicate that lipoprotein uptake by sympathetic neurons is receptor-mediated. Many of these receptors belong to the LDLR superfamily of which the LDLR, which has been detected in PNS and CNS neurons (9, 16, 30, 57), is the prototype. Our data show that the LDLR is present in cell bodies/proximal axons and distal axons of rat sympathetic neurons, with an apparent enrichment in cell bodies. It is likely, therefore, that in our studies the internalization of hLDL was mediated primarily by the LDLR. However, since the LDLR only binds lipoproteins that contain apoB and/or apoE (55), this receptor probably does not participate in the uptake of hHDLapo, which contain neither apoB nor apoE.

A spatially distinct expression of the LDLR has been observed in other polarized cells. For example, in Madin-Darby canine kidney cells expression of recombinant LDLRs resulted in their preferential targeting to basolateral membranes (58). In addition, using adenovirus expression the LDLR was highly expressed in the somatodendritic region of hippocampal neurons but was undetectable in distal axons. In contrast, mutant LDLRs lacking a putative basolateral targeting sequence were most highly concentrated in growth cones and distal axons (59). The distribution of endogenous LDLRs was not examined in this report. However, Li et al. (60) reported the presence of a cell surface receptor that bound LDL with the characteristics of the LDL receptor in both apical and basolateral domains of Madin-Darby canine kidney cells. Studies on the sorting of viral proteins (61) and endogenously expressed proteins (62, 63) in hippocampal neurons and Madin-Darby canine kidney cells have suggested that neurons and polarized epithelial cells share common mechanisms of protein targeting and sorting, with the axonal membrane being equivalent to the apical domain of epithelial cells and the somatodendritic region being
Expression of the LDLR in many cell types is regulated by cellular cholesterol levels (55); when cholesterol synthesis is inhibited, expression of LDLRs increases. In the present study, we inhibited cholesterol synthesis in sympathetic neurons and found that the amount of the LDLR in cell bodies/proximal axons, but not in distal axons, increased. Spatially independent regulation of expression of the LDLR has been previously observed in other polarized cells. For example, the number of LDLRs is regulated by cellular cholesterol content on only basolateral, but not apical, membranes of Madin-Darby canine kidney cells (60). One possible explanation for the lack of up-regulation of expression of the LDLR in distal axons in response to pravastatin is that the reduced cholesterol content might impair anterograde vesicular transport that would prevent LDLRs, which are synthesized in the cell bodies, from being exported to distal axons.

Other Lipoprotein Receptors in Sympathetic Neurons—Several members of the LDLR superfamily have been proposed to be involved in neuronal metabolism and pathogenesis of neurodegenerative diseases (29). For example, LRP, a multifunctional receptor that mediates the endocytosis of several ligands (64), is expressed in brain and spinal cord (18), as well as in the somatodendritic domain of cultured hippocampal neurons (22). The LRP and one of its ligands, apoE, have been reported to play an important role in early hippocampal development (19). Moreover, apoE synthesis dramatically increases in the CNS and PNS after nerve injury (10, 12, 46), and apoE-containing lipoproteins have been proposed to play a role in peripheral nerve regeneration (8) and dendritic remodeling in the CNS (65). Consequently, we were surprised that we could detect no LRP in sympathetic neurons. Our result is, however, consistent with the idea that CNS and PNS neurons might express distinct populations of lipoprotein receptors because the types of lipoproteins to which they would be exposed in their native environments are different.

LR11 is a recently discovered member of the LDLR superfamily (23–25). In mammals, LR11 is abundant in the brain and binds apoE-containing lipoproteins (26). This receptor has been suggested to play a role in lipoprotein metabolism in the CNS (25). We found that LR11 is present in cell bodies/proximal axons of sympathetic neurons but, interestingly, not in distal axons.

Our experiments also show that LR7/8B, for which apoE is a ligand, is expressed throughout cell bodies and axons of sympathetic neurons. cDNAs encoding this receptor have been cloned from brain of human, mouse, and chicken (27, 28). Interestingly, in chickens, which lack apoE, LR11 and LR7/8B are abundant in the brain. The importance of LR7/8B in CNS development is underscored by the finding that mice lacking both LR7/8B and the very low density lipoprotein receptor (another LDLR family member) (66) display an inversion of cortical layers during development (67, 68). Some receptors of the LDLR superfamily have recently been implicated in intracellular signaling events because their cytosolic domains interact with signaling molecules such as mDab1 (68). Whether or not these receptors function in vivo for endocytosis of lipoproteins and/or as signaling receptors is not yet known.

Uptake of Chicken LDL—cHDL were used as model lipoproteins because they exhibit some similarities to mammalian LDL. Although chickens lack apoE, their HDL contain apoAI that has been proposed to be a "surrogate" for mammalian apoE (47, 69, 70). Both mammalian apoE and chicken apoAI are actively expressed during development and myelination of sciatic nerves (48, 71), and synthesis of these apoproteins increases dramatically after peripheral nerve injury (46). Our data show that cHDL are internalized by sympathetic neurons in a manner analogous to that of hLDL but unlike that of hHDL3.

Uptake of hHDL3 by Distal Axons—The finding that hHDL3 are internalized by distal axons is intriguing although the distribution of lipoprotein receptors that we report here does not explain how this uptake occurs. Presumably, other receptors are present that mediate the uptake of hHDL3 by distal axons. Since cell bodies did not internalize either lipids or proteins of hHDL3, our data imply that cell bodies lack a functional hHDL3 receptor. The best characterized HDL receptor is SR-BI (72). SR-BI binds HDL via apoAI (73) and mediates a selective uptake of lipids, particularly cholesteryl esters, from HDL (52, 74–76) and LDL (77, 78), without a concomitant uptake of apoproteins. In rat adrenal cells, the selective uptake of cholesteryl esters occurs by a mechanism distinct from the classical endosomal/lysosomal pathway (79). Similarly, in rat hepatoma cells HDL cholesteryl esters are hydrolyzed extralysosomally (80). SR-BI is abundantly expressed in liver and steroidogenic tissues but has not been found in brain (81), and we were unable to detect SR-BI in sympathetic neurons. In addition to selective lipid uptake from HDL, the endocytosis and lysosomal degradation of HDL holoparticles have previously been observed (53, 54). In our experiments, not only lipids but also proteins of hHDL3 were internalized by axons and transported to cell bodies, indicating holoparticle uptake. However, our data are also consistent with the possibility that distal axons participate in a selective uptake of some lipids from HDL, in addition to holoparticle uptake.

Since hHDL3 lack apoE they are unlikely to be internalized by the LDLR although receptors participating in the uptake of intact HDL particles have not yet been clearly defined. Recently, cubulin has been identified as a receptor that mediates the endocytosis/lysosomal degradation of HDL (82, 83). Since cubulin lacks a membrane-spanning domain, it is considered to be a peripheral membrane protein (84) that requires a co-receptor, probably megalin, to mediate endocytosis (82). Megalin (also known as gp330 or LRP2) is a member of the LDLR family and binds multiple ligands including apoE and apoJ (reviewed in Ref. 64). This finding is of particular interest in relation to cholesterol homeostasis in the brain and during PNS nerve regeneration since lipoproteins involved in those processes are thought to contain apoE and/or apoJ (32). Cubulin is localized to apical membranes of polarized endothelial cells of the kidney (85), and megalin is also expressed on the apical surfaces of many epithelial cells (86, 87). Therefore, since neurons are polarized cells in which the distal axons are, in some ways, equivalent to the apical domain of other polarized cells (62, 63), one might predict that if megalin and cubulin were present they would be concentrated in distal axons. If this were the case, megalin and cubulin would be viable candidates for mediating hHDL3 uptake by distal axons. Whether or not cubulin and megalin are present in distal axons, and absent from cell bodies, of sympathetic neurons warrants further investigation.

In summary, we have shown that cultured rat sympathetic neurons internalize lipids and proteins of lipoproteins by receptor-mediated mechanisms. Although LRP was absent from these neurons, related receptors such as the LDLR, LR7/8B, and LR11 were detected. These receptors were differentially distributed, and expression of the LDLR was differentially regulated in cell bodies and distal axons of sympathetic neurons. Our studies also show that distal axons, but not cell bodies, of sympathetic neurons have the capacity to internalize intact HDL particles that lack apoE.

Acknowledgments—We thank Russ Watts and Grace Martin for ex-
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J. Biol. Chem. 2000, 275:19883-19890. doi: 10.1074/jbc.275.26.19883

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