Low-density Lipoprotein Receptor-Related Protein (LRP) Mediates the Endocytosis of Anionic Liposomes in Neurons

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Running Title: CONSTITUTIVE ENDOCYTOSIS OF ANIONIC LIPOSOMES IN NEURONS
SUMMARY

We have recently demonstrated that anionic liposomes efficiently introduce foreign DNA into postmitotic neurons and other cell types. To investigate the mechanism of liposome uptake, we followed the internalization of anionic liposome-encapsulated Cy3-labeled oligonucleotides (AL-Cy3ONs) by hippocampal neurons using confocal microscopy. Uptake of AL-Cy3ONs was widespread and time- and temperature-dependent, indicative of receptor-mediated endocytosis. The low-density lipoprotein receptor-related protein (LRP) was crucial for anionic liposome endocytosis because the receptor-associated protein or an LRP antibody inhibited internalization and fibroblasts lacking LRP did not internalize AL-Cy3ONs. Using selective endocytosis inhibitors, we found that liposome endocytosis and intracellular transport required clathrin, dynamin, an intact cytoskeletal network, and phosphatidylinositol 3-kinase activity. Cy3ONs did not significantly colocalize with recycling endosomal/lysosomal markers and entered neuronal nuclei within 1-3 h of incubation. Approximately 50% of the internalized liposomal phospholipids were recycled back to the cell surface, in keeping with the fluidity of their acyl chains. Liposome endocytosis did not require heparan sulfate proteoglycans (HSPG) or cause calcium influx into neurons. Thus constitutive endocytosis of anionic liposomes by LRP, utilizes only one component in contrast to the more involved HSPG-LRP pathway implicated in the pathogenesis of Alzheimer’s disease.
ENDOCYTOSIS IN NEURONS

INTRODUCTION

Endocytosis in neurons has mainly been studied in the context of synaptic vesicle recycling (1) and the regulation of neurotransmitter receptor numbers in the post-synaptic membrane (2). Constitutive endocytosis also occurs in neurons, albeit at a slower rate than in nonpolarized or mitotically active cells. Growth factors and hormones modulate the rate of constitutive endocytosis of neurotransmitter receptors as well as receptors involved in nutrient acquisition and metabolism. Neurotrophins such as NGF¹ and BDNF increase both the recruitment of clathrin to the plasma membrane and the rate of constitutive endocytosis of transferrin in hippocampal neurons (3).

Little, however, is known about the mechanisms of internalization of exogenous macromolecules such as phospholipids and nucleic acids in neurons. Insight into the interactions between neurons and these molecules would further our understanding of lipid and DNA transport pathways and provide potential therapeutic advantages. We have recently designed and developed an anionic liposome vector (composed of the anionic phospholipid DOPG and the zwitterionic phospholipid DOPC) for oligonucleotide delivery to neurons. Antisense oligonucleotides (ONs) targeted to the p53 tumor suppressor mRNA, delivered to neurons via anionic liposomes, efficiently protected hippocampal neurons from excitotoxic death by sequence-specific down-regulation of p53 protein expression (4). Preliminary studies indicated that the uptake of Cy3-labeled oligonucleotides (Cy3ONs) encapsulated in anionic liposomes was rapid and widespread.

The present study was undertaken to delineate the mechanism of liposome internalization in neurons. Cy3ONs were encapsulated in anionic liposomes and the
uptake of these molecules by cultured rat hippocampal neurons was studied by confocal microscopy. Each stage in the endocytic pathway was retarded by biochemically interfering with specific proteins to determine the role of that protein in the internalization of liposomes. Here we demonstrate that anionic liposomes utilize constitutive endocytosis of the low-density lipoprotein receptor-related protein (LRP) to enter neurons followed by intracellular transport and processing via a classical endocytic pathway. In addition to providing a mechanistic basis for ON delivery by anionic liposomes, our results have also identified an efficient phospholipid transport pathway in neurons. This pathway appears to be a previously unrecognized, independent component of the more involved LRP-mediated endocytic pathway that has been implicated in the processing of the amyloid precursor protein and the pathogenesis of Alzheimer’s disease.
EXPERIMENTAL PROCEDURES

_Oligonucleotides and Liposomes-_ An antisense oligonucleotide to the p53 mRNA was designed as previously described (4). Oligonucleotides were synthesized and labeled at the 5’- end with Cy3 and purified by reverse-phase HPLC to remove free dye (Integrated DNA Technologies). The oligonucleotides were reconstituted in sterile, nuclease-free Tris-EDTA buffer (pH 7.2) and stored at –20°C. Encapsulation of ONs in anionic liposomes (87.5 mole percent DOPC and 12.5 mole percent DOPG), their characterization and complexation of ONs with cationic liposomes were all performed as previously detailed (4). Liposomes labeled with N-Rh-DOPE were prepared in a manner identical to that described for liposomes encapsulating Cy3ONs except that the lipid films contained 1 to 2.5 mole percent N-Rh-DOPE and the liposomes were prepared with buffer alone (i.e., without ONs).

_Cell Culture and Treatments-_ Primary cultures of hippocampal neurons were prepared from neonatal rat pups (P1 or P2) as previously described (4,5) and cultured on 22-mm square glass coverslips or 8-chambered glass slides (LabTek II, Nalgene Nunc) in serum-free Neurobasal medium with B27 supplements unless otherwise stated. Neurons were incubated with 2 µM Cy3ONs either free, encapsulated in anionic liposomes or as complexes with cationic liposomes for 30 min at 37°C unless stated otherwise. For low temperature studies, neurons were incubated at 4°C with AL-Cy3ONs for 10 minutes. The LRP-deficient mouse embryonic fibroblast cell line PEA-13 and its wild-type counterpart MEF-1 (6) were obtained from the American Type...
Culture Collection. These cells were cultured on 8-chambered glass slides in DMEM containing penicillin and streptomycin with 10% Cosmic calf serum (HyClone) and incubated with AL-Cy3ONs for either 1 or 3 h at 37°C. At the end of the incubation periods, uninternalized ONs, liposomes or complexes were removed by several washes with L15 (Leibovitz's) medium and fixed in 4% paraformaldehyde.

The following agents were used to manipulate specific steps of the endocytic cycle: 0.45 M hyperosmolar sucrose (ICN biochemicals), 1 µM FK506 (Calbiochem), and 100 nM wortmannin, 5 µg/ml nocodazole, 10 µg/ml cytochalasin D, 100 µg/ml heparin and 100 µg/ml protamine sulfate, (all from Sigma-Aldrich). The LRP inhibitor, receptor-associated protein (RAP), and the LRP blocking antibody R2629 were used at concentrations of 500 nM (7) and 100 µg/ml (6), respectively. Cells were treated with the drugs, RAP or R2629 for 10 minutes prior to incubation with AL-Cy3ONs for 30 minutes at 37°C, fixed and imaged.

*Colocalization Experiments*- Intracellular fates of endocytosed liposomes and ONs were determined by comparing their intracellular distributions with that of Oregon Green488-Transferrin (OG-Tf) and Alexa488-dextran (Molecular Probes) used as markers for the recycling and lysosomal compartments, respectively. Neurons were incubated with AL-Cy3ONs and either 100 µg/ml OG-Tf, for 30 minutes, 1 or 3 h; or with 1 mg/ml Alexa488-dextran for 3 h. For phospholipid transport experiments, neurons were incubated with liposomes labeled with N-Rh-DOPE and either OG-Tf, for 30 minutes, 1 or 3 h; or with 1 mg/ml Alexa-dextran for 3 h. These time points were chosen based on earlier work on the kinetics of transferrin and dextran endocytosis in
hippocampal neurons (3,8,9). At the end of the incubation periods, cells were rinsed and fixed for imaging as described above.

Confocal Microscopy and Image Analysis- Imaging was performed on a Leica TCS 4D confocal microscope equipped with a mercury/xenon lamp and argon/krypton laser. Cells were excited using the 488 nm laser line to detect OG-Tf and Alexa488-Dextran and the emitted fluorescence was collected using a 515 long-pass filter. The 568 nm laser line was used to excite Cy3 and N-Rh-DOPE (LP590 emission). Cells were imaged at a plane midway between the substrate-attached plasma membrane and the top of the cell, such that neuronal nuclei were clearly identifiable. In some cases, the entire volume of the cell was scanned in 0.5 µm increments. For experiments with Alexa488-dextran, planes in which the Alexa488 label was most visible were chosen to enable identification of late endosomal/lysosomal structures. Optimal images were obtained by averaging 16 images in the line-scan mode at the same fixed gains for all experiments. For the colocalization experiments, the cell outlines for each set of fields were traced out manually in the corresponding brightfield image and then used to mask the fluorescence images (Metamorph, Universal Imaging). In each cell, the total fluorescence intensity was measured and the percent of Cy3 label or rhodamine label that colocalized with the Oregon Green488 or the Alexa488 labels was calculated. All the representative fluorescence images shown in the figures were equally contrast enhanced using Adobe Photoshop® (Adobe).
Calcium Imaging - Hippocampal neurons cultured on 35-mm glass-bottomed petridishes (5) were loaded with 4 µM fura-2-AM (Molecular Probes) for 30 min. The cultures were mounted in recording solution containing (in mM) 139 NaCl, 3 KCl, 10 NaHEPES, 1.8 CaCl2, 0.8 MgCl2, 5 glucose, 15 sucrose, 0.1 glycine, pH 7.4, and on the stage of an inverted Nikon Diaphot microscope. The recording solution also contained 1µM tetrodotoxin to inhibit spontaneous firing of the neurons. Images were captured with a 40x objective every 20 seconds (Metafluor, Universal Imaging) using the following wavelengths: fura 2 - excitation 340±15 nm and 380±15 nm attenuated with a 10% quartz neutral density filter, emission 525 ± 25; N-Rh-DOPE, 565 ± 15 nm excitation, > 590 nm emission (10). All images were corrected by subtracting a wavelength-specific background from an unpopulated portion of the dish. In preliminary experiments, addition of recording solution or liposomes by manual pipetting caused a transient increase in intracellular Ca2+. To avoid this artefact, anionic liposomes labeled with N-Rh-DOPE were perfused onto the neurons at the rate of 1 ml/min for 10-15 min.

Protein binding to liposomes - Neurons were incubated with 100 µl liposomes in recording solution (as above) for 3 h with or without 500 nM RAP. The medium overlying the cells was first centrifuged at 320x g for 8 min to remove cell debris, followed by centrifugation at 100,000x g for 15 min at 25°C in a Beckman Ultracentrifuge to recover the liposomes (11). The liposomal pellet was resuspended in 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl and an aliquot was analyzed using the highly sensitive CBQCA protein assay kit (Molecular Probes) to determine if any proteins secreted into the medium bound to liposomes. Fluorescence associated
with any protein bound to the liposomes, labeled by the CBQCA reagent was detected using a Perkin Elmer LS55 fluorimeter.
RESULTS

Neuronal Uptake of Anionic Liposomes Occurs by Clathrin-Mediated Endocytosis-
Incubation of hippocampal neurons with anionic liposomes containing 2 µM Cy3ONs for 30 minutes at 37°C resulted in the localization of the labeled oligonucleotides in vesicular cytoplasmic structures but not in the nucleus (Fig. 1a). After a one-hour incubation, diffuse Cy3 fluorescence was observed in the nucleus (Fig. 1b). The intensity of the diffuse nuclear label increased after three hours and portions of the cytoplasm often contained uniform Cy3 fluorescence, in addition to the punctate label (Fig. 1c). Virtually all the neurons examined under the microscope and/or imaged exhibited Cy3 fluorescence 30 minutes after incubation with anionic liposomes. Uptake of anionic liposomes was greatly reduced at 4°C with Cy3 fluorescence seen only at the cell surface, indicating binding of liposomes to the plasma membrane but not internalization (Fig. 1d). The time- and temperature-dependent uptake of anionic liposomes containing Cy3ONs suggested that liposome internalization into neurons occurred by endocytosis and was possibly receptor-mediated. To identify the cellular elements required for liposome uptake, we inhibited various steps in the endocytic pathway. Figure 2 depicts the incidence of intracellular Cy3 fluorescence in neurons following various experimental manipulations.

Cell-surface receptors concentrated in clathrin-coated pits mediate the endocytosis of many macromolecules. To determine if clathrin-coated pits were involved in the uptake of anionic liposomes, hyperosmolar sucrose was used to disrupt clathrin assemblies (12). Treatment of neurons with 0.45 M sucrose completely prevented internalization of anionic liposomes containing Cy3-oligonucleotides (Fig. 3b) compared
to cells treated with AL-Cy3ONs alone (Fig. 3a). This confirmed that neuronal uptake of liposomes was receptor-mediated because hyperosmolarity inhibits receptor-mediated endocytosis, but does not interfere with non-specific fluid phase endocytosis (13).

Clathrin-dependent endocytosis involving accessory proteins such as the GTPase dynamin, amphiphysin and synaptojanin plays a critical role in synaptic vesicle recycling at nerve terminals (14). Dynamin and amphiphysin need to be dephosphorylated by the Ca\(^{2+}\)/calmodulin-dependent phosphatase, calcineurin, in order to interact with one another and the lipid bilayer (15). We used a calcineurin inhibitor, FK506, to study the role of dynamin in anionic liposome internalization. Treatment of neurons with FK506 prior to the addition of AL-Cy3ONs significantly decreased liposome endocytosis (Fig. 3c), providing further evidence that liposomes were internalized in neurons via clathrin-coated pits.

**Liposome Endocytosis Occurs Via the LDL Receptor-Related Protein** - The low-density lipoprotein receptor-related protein (LRP), a member of the LDL receptor gene family, is highly expressed in the mammalian central nervous system and has been implicated in the endocytosis of several unrelated ligands (7). A major function of lipoprotein receptors is the regulation of lipoprotein uptake and metabolism (16). Immunofluorescence staining with an antibody to LRP confirmed that this receptor was highly expressed in our hippocampal neuronal cultures (see Supplementary Material). To determine if LRP was involved in the endocytosis of anionic liposomes, we blocked LRP using the receptor-associated protein (RAP) which is a potent inhibitor of all known ligand interactions of LRP (7). RAP binds with high affinity to the heavy chain of LRP on
multiple ligand-binding domains and induces a conformational change in the receptor, thus interfering with ligand binding (17). When neurons were incubated with AL-Cy3ONs in the presence of RAP, both binding and internalization of anionic liposomes were inhibited. Note the complete absence of Cy3 fluorescence either on the cell surface or within the RAP-treated neurons (Fig. 4b) compared to those treated with AL-Cy3ONs alone (Fig. 4a). Further evidence for the involvement of LRP in anionic liposome endocytosis was obtained when preincubation of neurons with a neutralizing LRP antibody R2629 (6) also prevented AL-Cy3ON uptake (Fig. 4c).

Lastly, we compared the endocytosis of AL-Cy3ONs in immortalized mouse embryonic fibroblast cell lines that either expressed LRP (MEF-1) or lacked the receptor (PEA-13). After a 1-h incubation, almost all MEF-1 cells displayed robust Cy3 fluorescence (Fig. 5a) in contrast to the faint signal seen in PEA-13 cells (Fig. 5b). Following a 3-h incubation, Cy3 fluorescence was visible in the PEA-13 cultures at lower intensity than that seen in the MEF-1 cells, indicating that liposomes were being taken up by the PEA-13 cells, albeit with very slow kinetics. In contrast to the MEF-1 cultures where all the cells examined had the Cy3 label, only 50-60% of the PEA-13 cells exhibited Cy3 fluorescence after 3 h (compare Fig. 5c with 5d).

Endocytosis of AL-Cy3ONs Via LRP is Independent of HSPGs and Does Not Alter Neuronal Calcium Currents- Several LRP ligands, including $\alpha_2$-macroglobulin ($\alpha_2$-M), apolipoprotein E (apoE) and HIV Tat protein bind heparan sulfate proteoglycans (HSPGs) on the cell surface prior to being internalized by LRP. Consequently, RAP does not inhibit binding of HIV Tat to the plasma membrane but inhibits its
internalization and subsequent degradation (18). To study whether HSPGs were necessary for liposome endocytosis by LRP, we incubated neurons with AL-Cy3ONs along with 100 µg/ml each of heparin or protamine sulfate. Heparin is a specific inhibitor of HSPG and protamine competes with LRP ligands for HSPG binding sites (19). Neither heparin (Fig. 2 and 6a) nor protamine (Fig. 2) altered the level of Cy3 fluorescence within neurons after 30 minutes of incubation, indicating that HSPGs were not required for anionic liposome endocytosis by LRP.

To determine if endogenous proteins secreted by neurons could bind liposomes and act as intermediaries between liposomes and LRP, we measured protein binding to liposomes after a 3-h incubation with neurons. Incubations were carried out either in the absence or presence of 500 nM RAP to increase the possibility of protein-bound liposomes being recovered from the medium. The amount of protein detected by the CBQCA assay did not significantly differ between the untreated or RAP-treated controls and liposome-treated conditions (one-way ANOVA, p=0.5, Table I). As a positive control, liposomes were incubated with poly-L-lysine (Lysine/lipid phosphate charge ratios of 0.6 and 2) and 100% of the added polylysine was detected in the liposome pellet (not shown). As the amine moiety on the choline headgroup of DOPC was found to interact with the CBQCA dye, standard curves with BSA were constructed in solutions containing liposomes and the samples were diluted to minimize lipid interference. We were able to detect 10 nanograms of exogenously added BSA (not shown) indicating that within the limits of sensitivity of this assay, no endogenous proteins from cultured neurons bound liposomes.
Recent studies on cortical neurons and hippocampal slices have suggested a role for LRP in synaptic neurotransmission (20,21). Addition of activated α2-M (α2-M*), α2-M*) to cortical neurons caused a Ca2+ influx that was both spatially and temporally discrete. Only ligands that bind LRP at multiple sites were capable of eliciting this calcium response indicating that receptor dimerization was essential. To examine whether the endocytosis of anionic liposomes via LRP caused Ca2+ influx into neurons, we studied neuronal calcium currents during a continuous perfusion of liposomes labeled with N-Rh-DOPE. Anionic liposomes did not evoke a calcium response (Fig. 6b) although they were endocytosed as evidenced by rhodamine fluorescence in the neurons after liposomal perfusion (Fig. 6c).

Intracellular Trafficking of Anionic Liposomes is Associated with the Cytoskeleton and Requires PI 3-Kinase Activity- Microtubule-dependent movement is a predominant means of axonal and dendritic transport in neurons (22). To determine if intracellular trafficking of AL-Cy3ONs requires an intact microtubule network, we used nocodazole to depolymerize microtubules in hippocampal neurons. When neurons were incubated with anionic liposomes in the presence of nocodazole, Cy3 label was found only at the edges of the cell and on the plasma membrane (Fig. 7b).

Although an actin-based framework is required for the organization of clathrin-coated pits at the cell surface, the role of actin in receptor-mediated endocytosis is still unclear (23,24). The involvement of the actin cytoskeleton in liposome endocytosis was studied using cytochalasin D to depolymerize actin filaments. In contrast to nocodazole-treated neurons, no Cy3 fluorescence was detected in neurons incubated
with AL-Cy3ONs in the presence of cytochalasin D (Fig. 7c). This confirms previous reports that the cytochalasin D-sensitive step precedes the nocodazole-sensitive step in receptor-mediated endocytosis (25).

Activation of the phosphatidylinositol 3-kinase (PI 3-kinase) family of lipid kinases is involved in the rearrangement of cytoskeletal proteins, vesicle sorting and receptor recycling during endocytosis. Specific inhibitors such as wortmannin have been widely used to study the potential sites of PI 3-kinase function in the endocytic pathway (26). To determine if PI 3-kinase activity was necessary for neuronal endocytosis of anionic liposomes, neurons were incubated with wortmannin prior to the addition of AL-Cy3ONs. A low-level of cell-associated Cy3 fluorescence was observed in neurons pretreated with wortmannin for 10 minutes (Fig. 7d) and increasing wortmannin exposure time beyond 10 min not only abolished the internalization of liposomes but also caused formation of vacuoles associated with the plasma membrane (not shown). Other studies have also documented a temporal correlation between exposure to wortmannin and drastic changes in organelle morphology (27).

Cytoplasmic Cy3ONs Does Not Significantly Colocalize with Organelles Containing Transferrin or Dextran- To determine the identity of vesicular structures containing the Cy3 label, we incubated neurons with AL-Cy3ONs along with either Oregon Green 488-transferrin as a marker for the recycling endosomal pathway or Alexa 488-dextran as a fluid-phase marker for the lysosomal degradative pathway, for different time periods (Fig. 8, a & b and Table II). After 30 minutes of co-incubation, 15% of the total intracellular Cy3 label was present in the same organelles as transferrin. The
proportion of total Cy3 that colocalized with transferrin did not increase beyond 25% even after three hours of incubation. Only 20% of the total cell-associated Cy3 was present in compartments containing dextran. The lack of significant colocalization between Cy3ONs and transferrin suggested that either Cy3ONs do not undergo recycling, or that recycling does occur, but with kinetics that are far slower than that of transferrin. As only 20% of Cy3ONs were present in lysosomal compartments after 3 hours, the bulk of the cargo delivered by the endocytosed anionic liposomes was available to the cell.

**Liposomal Lipids are Preferentially Sorted Into Recycling Compartments** - Recent evidence suggests that lipids endocytosed from the plasma membrane are sorted into either recycling endosomes or late endosomes based on the length and degree of unsaturation in their acyl chains (28). To determine if the dioleoyl (two 18-carbon acyl chains with one cis-double bond each) phospholipids DOPC and DOPG used in our studies were sorted according to this model, we used the headgroup-labeled lipid, N-Rh-DOPE to tag the liposomes. As headgroup-labeled lipids do not spontaneously transfer between membrane leaflets, they can be expected to label liposomal lipids reliably during membrane trafficking after internalization (29). Fluorescence resonance energy transfer measurements between unlabeled and N-Rh-DOPE-labeled liposomes demonstrated that self-quenching of rhodamine within the bilayer was relieved only by calcium-induced liposome aggregation and fusion and not by simple mixing of labeled and unlabeled liposomes (not shown). Neurons were incubated with N-Rh-DOPE liposomes and the recycling endosomal or lysosomal markers for various time periods
(Fig. 8, c & d). Approximately 50% of the internalized liposomal lipid colocalized with transferrin, suggesting that the fluid nature of the phospholipids used in this study led to their preferential sorting into recycling compartments (Table II).
DISCUSSION

Trafficking of anionic liposomes in hippocampal neurons- A classical endocytic pathway involving LRP was responsible for the uptake and intracellular transport of anionic liposomes (see Supplementary Material for a schematic of the liposome uptake pathway). Three lines of evidence indicate that LRP is crucial for liposome endocytosis (Figs. 4 and 5): pretreatment of neurons with (i) RAP or (ii) a neutralizing LRP antibody inhibited binding and subsequent internalization of liposomes and (iii) uptake of AL-Cy3ONs in fibroblasts lacking LRP was markedly reduced compared to those expressing the receptor. While RAP is known to block ligand binding to all members of the LDL receptor family, the neutralizing antibody is specific for LRP. Further, previous studies have demonstrated no difference between the LRP-expressing and LRP-deficient fibroblasts in LDL receptor function (6), indicating that LRP, and not another member of the LDL receptor family, was responsible for anionic liposome uptake.

Internalization of the receptor-liposome complex was inhibited at low temperatures (Fig. 1) because interaction of the receptor’s cytoplasmic internalization signal with the clathrin adaptor protein AP2 is temperature-sensitive (30). Random dispersal of receptors on the membrane by treatment with hyperosmolar sucrose (12) or interfering with scission of the coated pit by treatment with FK506 (15) both inhibited anionic liposome endocytosis (Fig. 3). An intact cytoskeleton and PI 3-Kinase activity were also required for anionic liposome endocytosis (Fig. 7). Treatment with wortmannin greatly reduced Cy3 fluorescence in neurons, in agreement with reports that recruitment of LRP to the cell surface from endosomal storage pools was almost completely inhibited by wortmannin (31).
Colocalization experiments with endosomal/lysosomal markers indicated that Cy3ONs were neither recycled nor rapidly degraded within neurons (Fig. 8a & b). Alternatively, ON recycling may occur with slower kinetics than that of transferrin, like GPI-anchored proteins that are recycled approximately three times more slowly than transferrin (32). The length and fluidity of the acyl chains determined intracellular fate of liposomal phospholipids (Fig. 8c & d). The dioleoyl lipids used in our study preferentially partitioned into fluid membrane domains with concave curvature and were sorted into transferrin-containing tubulovesicular recycling endosomes. The differential colocalization of ONs and liposomal lipids with transferrin indicated divergence in their intracellular paths 30-60 min after endocytosis. After receptor-ligand dissociation, proteins of the annexin family on the luminal surface of endosomes may bind to the anionic liposomes. Annexins cause lateral segregation of phosphatidylglycerol (PG) in mixed bilayers of phosphatidylcholine and PG in the presence of physiological concentrations of Ca^{2+} (33). Liposome destabilization by annexins would then provide a conduit for ONs into the cytoplasm from where they can freely diffuse into the nucleus, accounting for the differential sorting of the lipids and ONs.

**LRP-mediated endocytosis for macromolecule delivery to neurons** - In marked contrast to the rapid and widespread uptake of Cy3ONs delivered by anionic liposomes, neurons incubated with Cy3ONs either free or complexed with the cationic lipid DC-Chol/DOPE exhibited minimal intracellular Cy3 fluorescence (Fig. 2 and Supplementary Material). LRP is a receptor that is concentrated in coated pits in the absence of any stimulus (34) and is constitutively endocytosed, irrespective of ligand binding. In CHO cells, ~60% of
cell surface LRP is endocytosed within 5 min and ~50% of the internalized LRP is recycled within 30-60 min (35). The high sequence variability between the 31 ligand-binding sites of LRP endows different domains with unique charge densities and hydrophobic patches, resulting in distinct ligand-recognition sites (36,37). Initial binding of many LRP ligands with basic residues occurs via HSPGs which concentrate ligand on the cell surface and present it to LRP for internalization (38). In contrast, endocytosis of anionic liposomes was independent of HSPGs indicating that liposomes may interact directly with LRP (Fig. 6a). The observation of uptake during liposome perfusion in protein-free solution and the analysis of liposomes harvested after incubation with neurons (Fig. 6 and Table I) suggested that proteins either present in or secreted into the medium did not bind or mediate liposome endocytosis via LRP. The spatially restricted calcium influx reported in primary neurons following multivalent ligand binding to LRP (20) was not observed in our experiments suggesting that liposomes did not induce LRP multimerization and liposome endocytosis by LRP was distinct from that of other LRP ligands (Fig. 6b & c).

The widespread expression of LRP (39) should enable anionic liposomes to deliver nucleic acids and possibly, proteins, to a broad spectrum of cell types (4) and tissues. In light of recent evidence that stressful external stimuli increase the rate of endocytosis (40), the LRP-dependent pathway of anionic liposome uptake into neurons holds important implications for therapeutic approaches in a number of diseases.

**Physiological relevance of LRP-mediated phospholipid uptake in neurons** - It is well established that the synthesis and metabolism of cholesterol and phospholipids in the
CNS are compartmentalized from that in the plasma by the blood brain barrier (41). Astrocytes package cholesterol into apoE-containing lipoprotein particles that are then taken up by neurons through LRP-mediated endocytosis (42). Recent studies demonstrate that apoE lipoproteins, internalized via LRP, promote neurite outgrowth (43) and synaptogenesis (44). Further, Alzheimer’s disease (AD) is associated with altered phospholipid metabolism and marked reductions in the phosphatidylcholine content of synaptosomes and plasma membrane of AD hippocampi (45). Levels of apoE and phosphatidylcholine precursors increase in parallel with neurite sprouting in the lesioned hippocampus, probably enabling membrane synthesis and dendritic rearrangement (46). The importance of LRP-mediated endocytosis in the growth and maintenance of neuronal structural plasticity is underscored by the rapid mobilization of LRP to the plasma membrane from intracellular storage pools following treatment with NGF (47). Growth factor-induced increase in the endocytosis of lipoproteins may be a way of providing neurons with the lipids and proteins necessary for growth and regeneration.

LRP-ligand interactions and apoE-delivered cholesterol have been implicated in numerous intracellular signal transduction events (48). In addition, multivalent ligand binding to LRP causes local influx of Ca²⁺ (20) and affects synaptic neurotransmission (21) probably by triggering NMDA receptor clustering, because the cytoplasmic tail of LRP physically interacts with NMDA receptors via PSD95 (49). Modulation of downstream signaling cascades and neurotransmission by the apoE-HSPG-LRP pathway has been hypothesized to play a role in AD pathogenesis (48). However, our results demonstrate for the first time that constitutive endocytosis of liposomes by LRP
utilizes only one component of the LRP endocytic pathway, without involving HSPG or altering intracellular calcium levels. The existence of an endogenous phospholipid ligand in the CNS that might activate LRP-mediated endocytosis independent of HSPG remains to be determined.
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REFERENCES


FOOTNOTES

1 The abbreviations used are: α2-M, alpha 2-macroglubulin; Aβ, amyloid β peptide; AL-Cy3ON, anionic liposomes encapsulating Cy3-labeled oligonucleotides; ApoE, apolipoprotein E; BSA, bovine serum albumin; CHO, Chinese hamster ovary; Cy3ON, Cy3-labeled oligonucleotides; DC-Chol, dimethylaminoethane carbamoyl cholesterol; DOPE, dioleoyl phosphatidylethanolamine; DOPC, dioleoyl phosphatidylcholine; DOPG, dioleoyl phosphatidylglycerol; GPI, glycosylphosphatidylinositol; GTPase, guanosine triphosphatase; HSPGs, heparan sulfate proteoglycans; NMDA, N-methyl-D-aspartate; N-Rh-DOPE, Lissamine Rhodamine DOPE; LRP, low-density lipoprotein receptor-related protein; NGF, nerve growth factor; OG-Tf, Oregon Green 488-Transferrin; ONs, Oligonucleotides; RAP, receptor-associated protein.
FIGURE LEGENDS

FIGURE 1. An energy-dependent process is responsible for the internalization of AL-Cy3ONs. Intracellular Cy3 fluorescence was observed at (a) 30 min; (b) 1 h; and (c) 3 h after incubation at 37°C. Note the strong Cy3 fluorescence in neuronal nuclei in b and c. (d) Internalization, but not binding of AL-Cy3ON to the plasma membrane, was inhibited at 4°C. Scale bar: 5 µm.

FIGURE 2. Comparison of uptake of Cy3-labeled oligonucleotides encapsulated in anionic liposome across all experiments. Bars represent percent of imaged neurons (N = 45 to 120) containing punctate Cy3 fluorescence 30 min after the indicated incubation at 37°C unless otherwise stated. Final Cy3ON concentration was 2 µM in all experiments. AL-Cy3ON, anionic liposomes encapsulating Cy3-labeled oligonucleotides; 4°C, incubation performed at 4°C; Suc, 0.45 M sucrose; FK, 1 µM FK506; RAP, 500 nM receptor-associated protein; Hep, 100 µg/ml heparin; Prot, 100 µg/ml protamine sulfate; Noc, 5 µg/ml nocodazole; Wort, 100 nM wortmannin; Cy3ON, neurons incubated with Cy3ONs alone, without liposomes; pCL-Cy3ON, Cy3ONs complexed with cationic liposomes at a net-positive charge; nCL-Cy3ON, Cy3ONs complexed with cationic liposomes at a net-negative charge.

FIGURE 3. Internalization of AL-Cy3ONs proceeds by a clathrin-mediated, dynamin-dependent pathway. Pretreatment of neurons with (b) 0.45M sucrose or (c)
1 µM FK506 decreased the internalization of AL-Cy3ONs compared to (a) cells treated with AL-Cy3ONs alone. Scale bar: 5 µm.

FIGURE 4. Endocytosis of anionic liposomes is mediated by LRP. Pretreatment with (b) 500 nM RAP or (c) 100 µg/ml anti-LRP inhibited both binding and endocytosis of AL-Cy3ONs compared to (a) cells treated with AL-Cy3ONs alone. Scale bar: 5 µm.

FIGURE 5. Cell surface expression of LRP is essential for the rapid uptake of AL-Cy3ON. After a 1-h incubation with AL-Cy3ONs, Cy3 label was visible in (a) LRP-expressing MEF-1 cells but not in (b) LRP-deficient PEA-13 cells. Following a 3-h incubation, some Cy3 label was visible in (d) PEA-13 cells, although far less than that seen in (c) MEF-1 cells. Scale bar: 10 µm.

FIGURE 6. Anionic liposome endocytosis by LRP is independent of HSPG and does not alter neuronal calcium contents. (a) Neurons were treated with 100 µg/ml heparin prior to incubation with AL-Cy3ON. Scale bar: 5 µm. (b) Fura-2 ratios in hippocampal neurons were not altered during perfusion of anionic liposomes labeled with N-Rh-DOPE but increased in response to 100 µM NMDA. Data were from a representative field of 23 neurons from among 110 neurons imaged in 5 experiments. (c) Image taken at 24 min (*) indicated the uptake of N-Rh-DOPE within the same neurons in the field. An image taken at comparable gains and wavelengths prior to the anionic liposome perfusion was blank (not shown).
FIGURE 7. **AL-Cy3ON endocytosis requires an intact cytoskeleton and PI 3-kinase activity.** Neurons pretreated with (b) 5 µg/ml nocodazole, (c) 100 µg/ml cytochalasin D or (d) 100 nM wortmannin exhibited very low levels of Cy3 fluorescence compared to (a) cells treated with AL-Cy3ONs alone. Scale bar: 5 µm.

FIGURE 8. **Cy3ONs delivered by anionic liposomes are neither significantly recycled nor degraded while the liposomal lipids are sorted into recycling compartments.** Neurons were incubated with a, Cy3ONs (red) and OG-Tf (green) for 1 h; b, Cy3ONs (red) and Alexa488-dextran (green) for 3 h; c, N-Rh DOPE (red) and OG-Tf (green) for 1 h; and d, N-Rh DOPE (red) and Alexa488-dextran (green) for 3 h. Areas of colocalization of the probes appear yellow. Scale bar: 5 µm.
Table I. Analysis of endogenous protein binding to anionic liposomes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein recovered from liposome pellet (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>37.484 ± 11</td>
</tr>
<tr>
<td>Anionic liposomes</td>
<td>36.955 ± 10</td>
</tr>
<tr>
<td>500 nM RAP</td>
<td>44.911 ± 4.9</td>
</tr>
<tr>
<td>Anionic liposomes + 500 nM RAP</td>
<td>40.85 ± 13</td>
</tr>
</tbody>
</table>

Protein amounts were determined by the CBQCA assay as detailed in Experimental Procedures. Values are presented as Mean ± S.D., n = 3.
Table II. Colocalization of Cy3-labeled oligonucleotides (Cy3ONs) or rhodamine-labeled lipids (N-Rh-DOPE) with transferrin or dextran in hippocampal neurons.

<table>
<thead>
<tr>
<th>Markers</th>
<th>30 min</th>
<th>1 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3ONs &amp; Transferrin</td>
<td>14.86 ± 1</td>
<td>22.67 ± 1.7</td>
<td>24.62 ± 2</td>
</tr>
<tr>
<td></td>
<td>(n = 43)</td>
<td>(n = 46)</td>
<td>(n = 46)</td>
</tr>
<tr>
<td>Cy3ONs &amp; Dextran</td>
<td>N.D.</td>
<td>N.D.</td>
<td>20.13 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n = 46)</td>
</tr>
<tr>
<td>N-Rh-DOPE &amp; Transferrin</td>
<td>45.56 ± 2.2</td>
<td>51.88 ± 2.7</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>(n = 46)</td>
<td>(n = 43)</td>
<td></td>
</tr>
<tr>
<td>N-Rh-DOPE &amp; Dextran</td>
<td>N.D.</td>
<td>N.D.</td>
<td>22.18 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n = 43)</td>
</tr>
</tbody>
</table>

Quantitation of the extent of colocalization of Cy3ONs or N-Rh-DOPE with markers for recycling endosomes (transferrin) and lysosomes (dextran) was performed as detailed in Experimental Procedures. $n$, number of neurons imaged per condition in three separate experiments; N.D., not determined.
FIGURE S1. **Hippocampal neurons in culture express the low-density lipoprotein receptor-related protein.** Neurons were fixed in 4% paraformaldehyde and incubated with 30 μg/ml R2629 (rabbit polyclonal anti-LRP) for 2 h at room temperature, followed by FITC-conjugated goat-anti-rabbit IgG for 1 h. Imaging and image processing were performed as detailed in Experimental Procedures.

FIGURE S2. **Anionic liposomes are an efficient system for ON delivery to neurons.** Neuronal uptake of oligonucleotides in anionic liposomes was compared with that of free oligonucleotides and oligonucleotides complexed with cationic lipids. These cationic lipid-DNA complexes bind negatively charged cell membranes via electrostatic interactions and undergo non-specific endocytosis (1). The requirement for a net-positive charge on the complexes, initially held to be indispensable for complex internalization, is now being debated (2). We complexed cationic DC-Chol/DOPE liposomes with Cy3ONs at two different charge ratios such that the resulting complexes had either a net-positive or a net-negative charge. Incubation of hippocampal neurons with net-positive cationic lipid-Cy3ON complexes or with 2 μM Cy3ONs without a delivery vector, resulted in a low level of diffuse cellular fluorescence in a few cells (Panels b and c) compared to cells treated with AL-Cy3ONs (Panel a). Some of the neurons incubated with net-negative cationic lipid-Cy3ON complexes exhibited bright
fluorescence associated with the plasma membrane, with only sparse fluorescence observed inside occasional neurons (Panel d). Scale bar: 5 µm.

FIGURE S3. Proposed pathway of endocytosis and intracellular trafficking of anionic liposomes in hippocampal neurons. Antagonists to various steps in the endocytic process used in this study are indicated.

References:

Low-density lipoprotein receptor-related protein (LRP) mediates the endocytosis of anionic liposomes in neurons
Aparna Lakkaraju, Yueh-Erh Rahman and Janet M. Dubinsky

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