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Cholesterol Modifies Huntingtin Binding to, Disruption of, and Aggregation on Lipid Membranes

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Abstract

Huntington's disease (HD) is an inherited neurodegenerative disease caused by abnormally long CAG-repeats in the huntingtin gene that encode an expanded polyglutamine (polyQ) domain near the N-terminus of the huntingtin (htt) protein. Expanded polyQ domains are directly correlated to disease-related htt aggregation. Htt is found highly associated with a variety of cellular and subcellular membranes that are predominantly comprised of lipids. Since cholesterol homeostasis is altered in HD, we investigated how varying cholesterol content modifies the interactions between htt and lipid membranes. A combination of Langmuir trough monolayer techniques, vesicle permeability and binding assays, and in situ atomic force microscopy were used to directly monitor the interaction of a model, synthetic htt peptide and a full-length htt-exon1 recombinant protein with model membranes comprised of total brain lipid extract (TBLE) and varying amounts of exogenously added cholesterol. As the cholesterol content of the membrane increased, the extent of htt insertion decreased. Vesicles containing extra cholesterol were resistant to htt-induced

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Author Contributions

J.L. and S.L.F. conceived and coordinated the study and wrote the paper. X.G. performed all in solution AFM experiments on htt-exon1(51Q) and lipid bilayers and purified GST-htt-exon1(51Q). W.A.C. performed Langmuir trough experiments and the vesicle leakage assays with Nt¹⁷-Q35-P10-KK. M.C. and P.J. performed the TBLE/PDA lipid binding assays and assisted in purifying GST-htt-exon1(51Q). A.E.L. performed cholesterol concentration assays and assisted with other control experiments. All authors analyzed the results and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

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Additional supplemental data (PDF)

Supplemental movie 1 (MPG1)

Supplemental movie 2 (MPG2)

permeabilization. Morphological and mechanical changes in the bilayer associated with exposure to htt were also drastically altered by the presence of cholesterol. Disrupted regions of pure TBLE bilayers were grainy in appearance and associated with a large number of globular aggregates. In contrast, morphological changes induced by htt in bilayers enriched in cholesterol were plateau-like with a smooth appearance. Collectively, these observations suggest that the presence and amount of cholesterol in lipid membranes play a critical role in htt binding and aggregation on lipid membranes.

Graphical abstract

Huntington's disease, a neurodegenerative genetic disorder, is caused by an expanded polyglutamine (polyQ) domain near the N-terminus of the huntingtin (htt) protein.¹ PolyQ expansion promotes the self-assembly of htt into fibrils and other types of aggregates that accumulate in the hallmark inclusion bodies found in HD brain tissues.² Furthermore, age of onset and disease severity are strongly correlated with the length of the polyQ domain, with a critical expansion length of at least ~35 glutamines required for disease.³ Full-length htt is over 3000 amino acids long, but it undergoes proteolysis, resulting in a variety of truncation products.^{4,5} Specifically, N-terminal htt fragments, such as the first exon of htt, have been shown to have a potentially important role in HD. For example, expression of htt exon1 with an expanded polyQ tract causes a progressive neurological phenotype in transgenic mice.^{2,6} Furthermore, N-terminal fragments similar to exon1 are detected in knock-in mouse models expressing full length htt,⁷ and fragments on the order of exon1 have been detected in HD patients.⁵

While htt appears to be a multifunctional protein,^{8–12} there is considerable published support that it intimately interacts with a variety of lipid membranes.^{13–17} Htt localizes with brain membrane fractions¹⁸ and is essential for the normal development of several perinuclear membrane organelles, including mitochondria and the ER.^{19–21} As a result of its normal function, htt localizes to specific subcellular compartments⁸ and has been implicated in the transport of lipid vesicles (endocytic, synaptic or lysosomal), especially along microtubules.^{11,22–24} Furthermore, the first 17 amino acids of htt (Nt¹⁷) that flank the polyQ domain facilitate the binding of N-terminal fragments to lipid membranes.^{25–27} Additionally, polyQ length correlates with magnitude of htt insertion and disruption of lipid membranes.^{13,28}

Cholesterol is an important structural component of biological membranes and is involved in regulating the fluidity of lipid bilayers. The brain is the most cholesterol-rich organ in the

mammalian body, with ~23% of total body cholesterol. ~70% of this cholesterol is contained within the myelin sheaths and is a crucial element for proper neuron and astrocyte function.^{29,30} Cholesterol affects the functional properties of membrane-resident proteins (ion channels and transmitter receptors) as well as plays a role in signal transduction, synaptogenesis, and neurotransmitter release.^{31–33} These functions are attributed to its regulatory role of the membrane physical properties.^{34,35} The presence of cholesterol is necessary for the formation of lipid rafts, used for cellular communication and signal transduction, via hydrogen bonding with the sphingosine hydroxyl group of sphingomyelin and alignment of the hydrophobic planar core causing condensation of the fatty acid domains.^{36,37} Cholesterol in the brain is synthesized endogenously, due to its inability to cross the blood-brain barrier.

Abnormalities in cholesterol metabolism and homeostasis have been observed in cellular and animal models of HD, as well as in HD patient tissues.^{38–40} However, the specifics of altered cholesterol content associated with HD remains controversial. Numerous studies suggest a decrease in cholesterol levels in HD models;^{41–44} however, other reports appear to demonstrate an accumulation or increase in cholesterol.^{45–47}

The importance of htt aggregation in HD has long been appreciated, and lipid bilayers have been shown to heavily influence the aggregation of a variety of N-terminal htt fragments.²⁵ However, the impact of specific lipid components, such as cholesterol, on modulating htt aggregation is poorly understood. Here, we characterize the interaction of N-terminal htt fragments with total brain lipid extract (TBLE) lipid membranes that contain varying amounts of exogenously added cholesterol.

EXPERIMENTAL PROCEDURES

Peptide Preparation

The synthetic, model htt peptide used in this study contains 35 glutamines (which is sufficient for aggregation⁴⁸), a C-terminal polyproline (polyP) domain that is 10 residues in length, and the previously mentioned Nt¹⁷ on the N-terminus. This peptide is referred to as Nt¹⁷-Q₃₅-P₁₀-KK. The lysines were added to the C-terminus to aid in solubility. Nt¹⁷-Q₃₅-P₁₀-KK peptides were obtained via custom synthesis (Keck Biotechnology Resource Laboratory, New Haven, Connecticut). Disaggregation and solubilization of Nt¹⁷-Q₃₅-P₁₀-KK peptides were achieved using established protocols.⁴⁹ Briefly, peptide was dissolved overnight in a 1:1 mixture of trifluoroacetic acid (TFA, Acros Organics) and hexafluoroisopropanol (HFIP, Acros Organics) at a concentration of 0.5 mg/mL. After vigorous vortexing, a stream of N₂ was used to evaporate the solvent. To remove any residual solvent, samples were then placed in a Vacufuge concentrator for 3 h (Eppendorf, Hauppauge, NY), resulting in thin peptide films. These peptide films were resuspended in ultrapure water that had been adjusted to pH 3 with TFA to 200 μ M, which was diluted in phosphate-buffered saline (PBS) (10 mM phosphate, 140 mM NaCl, 2.7 mM KCl) (Life Technologies) to a final concentration of 20 μ M at a pH of 7.3.

Purification of GST-htt-exon1 Fusion Proteins

Glutathione S-transferase (GST)–htt exon1 fusion proteins were purified as previously described.⁵⁰ For experiments presented here, htt exon1 with 51 repeat glutamines was used. In short, the GST-htt fusion proteins were expressed by induction in *Escherichia coli* with isopropyl β -D-thiogalactoside at 30 °C for 4 h. The cells were lysed with lysozyme (0.5 mg/mL). Fusion proteins were purified from lysate by liquid chromatography (LPLC, BioRad) using a GST affinity column. Relevant fractions to collect and purity were determined by gel electrophoresis. Cleavage of the GST moiety was achieved by Factor Xa (Promega, Madison, WI). Cleavage initiates aggregation. Prior to the addition of the cleaving agent, solutions of fusion proteins were centrifuged at 20000g for 30 min at 4 °C to remove preexisting aggregates. Experiments were carried out in buffer A (50 mM Tris–HCl, pH 7.0, 150 mM NaCl). To ensure efficient GST cleavage, the GST-htt exon1 fusion protein and respective cleavage agents were incubated for 1 h on ice prior to their addition to any assay.

Liebermann-Buchard Assay

To determine the cholesterol content of the stock TBLE, a Liebermann-Buchard assay was performed.⁵¹ To prepare 100 mL of Liebermann-Buchard reagent, 10 mL of concentrated sulfuric acid was added to 60 mL of acetic anhydride with continuous stirring in an ice bath. Next, 30 mL of acetic acid and 0.6 g of anhydrous sodium sulfate were added. Standard cholesterol samples were used to calibrate. 0.5 mL of the TBLE stocks dissolved in chloroform were added to 5 mL of Liebermann-Buchard reagent, vortexed, and placed in a water bath at 35 °C for 10 min. Absorbance was measured at 620 nm using a GENESYS 20 Visible spectrophotometer (Thermo Scientific).

Langmuir Trough Configuration

All lipid monolayer isotherm and peptide insertion experiments were conducted in a Teflon Langmuir trough with symmetric automated Teflon barriers (large inverted microscopy model, NIMA Technologies, Coventry, England). Surface pressure at the air/buffer interface was measured with a stationary Wilhelmy balance (NIMA Technologies, Coventry, England). All measurements were collected at 30 ± 0.5 °C, and the trough temperature was maintained with a circulating heated water bath (Isotemp 3016D water circulator, Thermo Fisher Scientific).

Lateral Compression Experiments

Lipid monolayer isotherms were conducted to characterize monolayer phase behavior as a function of lipid-packing density and subsequent altering effects of cholesterol. Stock TBLE (Avanti Polar Lipids) was obtained in chloroform and used without further purification. Cholesterol (Sigma-Aldrich) was received in powder form and was dissolved in chloroform (Sigma-Aldrich). TBLE and cholesterol were mixed in different ratios to a total spreading concentration of 0.4 mg/mL and stored in glass vials at –20 °C. The lipid monolayer was formed by dropwise addition of spreading solution on the PBS (pH = 7.3) surface; chloroform was allowed to evaporate for 15 min. Because of the lack of available quantitative characterization information on TBLE lipid components, the area per molecule at the interface was calculated assuming an average TBLE molecular weight of 850 g/mol.

The barriers compressed the monolayer at 6 cm²/min and isotherm measurements in the form of surface pressure (mN/m) versus mean area per lipid molecule (Å²/molecule) were taken at 1 s intervals until monolayer collapse.

Peptide Insertion into a Lipid Monolayer

Insertion experiments were performed to measure the amount of Nt¹⁷-Q₃₅-P₁₀-KK peptide insertion into TBLE and TBLE/cholesterol monolayers. The Langmuir trough setup was identical to an isotherm experiment, but the lipid monolayer was compressed to a surface pressure of 30 mN/m to mimic the lateral density of a cellular bilayer⁵² and then the barriers were set to feedback control to maintain constant surface pressure. Lyophilized peptide was rehydrated in TFA/PBS as described above and immediately injected beneath the monolayer for a final peptide concentration of 900 nM. After 10 min, if no increase in average area per molecule to signify peptide insertion was observed, the surface pressure was reduced by 4 mN/m increments down to a minimum of 14 mN/m. Percent insertion was determined as a % increase in area $((A_{\text{final}} - A_{\text{init}})/A_{\text{init}}) \times 100$ at each surface pressure.

Preparation of Vesicles for Leakage Assay

Large unilamellar vesicles (LUVs) of TBLE and TBLE/cholesterol were prepared to determine leakage caused by htt peptide insertion. Lipid films, evaporated with a stream of N₂ from a chloroform stock, were hydrated in 70 mM calcein (Sigma) via 5 min of vortexing and 5 min of sonication. The vesicles went through seven freeze-thaw cycles in dry ice/ethanol and warm water baths promoting unilamellar vesicles, followed by 11 extruder filtrations through a 100 nm pore polycarbonate filter (Avanti). Excess dye was removed with size exclusion chromatography using Sephadex G-50 beads (Sigma-Aldrich) and ultrapure water eluent. 120 nm diameter size uniformity was confirmed with dynamic light scattering (Zetasizer ZS90 [Malvern Worcestershire, UK]). Lipid concentration was determined by a phosphorus assay adapted from Anderson and Rouser.^{53,54} Briefly, a lipid fraction (500 μL) and 8.9 N H₂SO₄ (225 μL) were vortexed and heated for 20 min at 155 °C. After cooling, 6% H₂O₂ (75 μL) was added and heated for an additional 40 min at 155 °C. After cooling, 1.95 mL of ultrapure water, 5% molybdic acid (225 μL), and 10% ascorbic acid (225 μL) were added, and the solution was placed in a boiling water bath for 10 min. Absorbance at 820 nm was measured using a Jasco V-550 UV/visible spectrophotometer (Jasco, Easton, MD) and compared to a KH₂PO₄ standard reference plot to calculate final lipid concentration.

Vesicle Leakage Assay

TLBE LUVs with exogenous cholesterol ranging from 0 to 16 wt % were exposed to Nt¹⁷-Q₃₅-P₁₀-KK peptide resulting in a range from 1:1 to 64:1 lipid:peptide molar ratios at 25 °C. Lyophilized peptides were hydrated as outlined above and used promptly after hydration to minimize presence of non-monomeric species. Calcein fluorescence at 515 nm (495 nm excitation) was measured at room temperature prior to peptide exposure and 1 min after peptide administration using a RF-1501 spectrofluorometer (Shimadzu, Columbia, MD). Vesicles were then completely lysed using 1% Triton X-100 (Sigma) to measure a maximum fluorescence. Vesicle leakage caused by peptide was calculated by

$$\% \text{relative fluorescence} = \frac{I_F - I_1}{I_T - I_1} \times 100 \quad (1)$$

where I_F is fluorescent intensity after peptide addition, I_1 is initial fluorescence of the LUVs, and I_T is total fluorescence after complete lysis.

TBLE/PDA Lipid Vesicle Protein Binding Assay

TBLE/polydiacetylene (PDA) lipid binding assays were performed to measure the interaction between htt-exon1(51Q) and lipid vesicles. TBLE/PDA assays were performed using previously reported protocols.^{55,56} In short, diacetylene monomers 10,12-tricosadiynoic acid (GFS Chemicals, Columbus, OH), TBLE, and an appropriate amount of cholesterol were dissolved in a solution of 1:1 chloroform/ethanol. Total lipid (TBLE + cholesterol) and PDA was always mixed in a 2:3 molar ratio. The solution was evaporated off in a rotary evaporator, leaving a thin, dry film. 1× Tris-buffered saline (TBS) heated to 70 °C was added to the film and sonicated for 5 min at 100 W using a sonic dismembrator (FisherSci). To ensure self-assembly of the vesicles, the suspension was stored at 4 °C overnight. The diacetylene monomers were polymerized by irradiation at 254 nm with 7 lm for 10 min (room temperature with stirring), resulting in a brilliant blue color. As controls, each TBLE/PDA vesicle sample was exposed to 1× Buffer A (negative control) or NaOH (pH 12) (positive control). Polymerized TBLE/PDA vesicles were exposed to htt-exon1(51Q) at a final concentration of 20 μM. Experiments were performed in triplicate in a 96-well format, and the colorimetric response of each well was recorded over 16 h using an Infinite M1000 Pro plate reader (TECAN, Switzerland) at 25 °C measuring both the blue component (640 nm) and the red component (500 nm) of the spectrum. A quantitative value representative of blue-to-red color transition was obtained by determining the % colorimetric response (%CR), defined as^{55,56}

$$\% \text{CR} = [(PB_0 - PB_1) / PB_0] \times 100 \quad (2)$$

where PB is a red/blue ratio of absorbance (A) defined as $A_{\text{blue}} / (A_{\text{blue}} + A_{\text{red}})$. A_{blue} is the absorbance at the “blue” component in the UV-vis spectrum (~640 nm); A_{red} is absorbance at the “red” component (~500 nm), PB_0 is the red/blue ratio of the control sample (before induction of color change), and PB_1 is the value obtained for the vesicle solution after addition of peptides.

AFM Imaging Conditions

In situ AFM experiments were performed with a Nanoscope V MultiMode scanning probe microscope (Bruker, Santa Barbara, CA) equipped with a closed-loop “vertical engage” J-scanner and a sealable tapping fluid cell. Images were acquired with silicon cantilevers (VISTAprobes) with nominal spring constants of ~0.1 N/m. Scan rates were ~2 Hz with cantilever drive frequencies ranging from ~8 to 10 kHz. The free amplitude of the cantilever was ~25 nm, and the tapping amplitude was set at 75% of free amplitude. The Matlab image

processing toolbox (Mathworks, Natick, MA) was used to analyze AFM images. Topographic and phase images were captured simultaneously. In phase imaging, the shift in the phase of the oscillating probe tip with respect to the oscillating drive is mapped, and these phase shifts highlight compositional contrast of the surface. In solution, phase-shifts are sensitive to the local elastic response of the sample and can be used to map local elasticity of samples like lipid membranes.⁵⁷

Preparation of Supported TBLE Bilayers for AFM Experiments

TBLE (Avanti Polar Lipids) and cholesterol were dissolved in chloroform (Fisher Scientific) and mixed at appropriate ratios to have samples of pure TBLE and TBLE spiked with 10%, 20%, or 30% exogenously added cholesterol based on mass. The chloroform was evaporated in the fume hood in the air. The resulting lipid films were resuspended in Buffer A (pH 7.3) at a concentration of 0.5 mg/mL. Bilayers and multilayer lipid sheets were formed by at least five sequential freeze–thaw cycles. The lipid suspensions were then sonicated for 7 or 8 min to promote vesicle formation. 35 μ L of the vesicle solution was injected directly into the AFM fluid cell and onto freshly cleaved mica. The formation of a continuous bilayer via vesicle fusion was directly observed by continual AFM imaging. AFM experiments were performed between 23 and 25 °C.

RESULTS

Addition of Cholesterol Reduces the Insertion of htt into Lipid Membranes

To understand how cholesterol affects interactions between htt and the cell membrane, monomeric peptide insertion into a TBLE monolayer with varying amounts of exogenous cholesterol was measured. The amount of cholesterol in the stock TBLE (no exogenous cholesterol) was 22 wt % as measured by a Liebermann-Buchard assay. This wt % of cholesterol with respect to total lipid content is similar to that found in the human brain.⁵⁸ When peptide is introduced into an aqueous solution, the peptide molecules partition between the bulk and the air/buffer interface until a certain surface pressure, the equilibrium spreading pressure, is attained. It has been previously shown that Nt¹⁷-Q₃₅-P₁₀-KK has appreciable surface activity (equilibrium spreading pressure or ESP of 17.3 mN/m at 900 nM) due to the inclusion of the N-terminal lipid binding domain, and this, along with the polyP domain, promotes insertion into pure TBLE lipid monolayers.²⁵ A TBLE lipid monolayer with either 0, 7, or 16 wt % exogenous cholesterol at the air/buffer interface, serving as a model for the outer leaflet of a cell membrane, was compressed to a physiologically relevant surface pressure of 30 mN/m,⁵² held at this pressure, and then freshly prepared peptide was injected into the subphase beneath the monolayer. Change in average area per molecule at the interface, which indicates peptide insertion, was monitored until the monolayer equilibrated. Then the pressure was stepped down by a 4 mN/m increment and the process was repeated. The addition of cholesterol to TBLE monolayers systematically decreased the amount of htt peptide insertion at each surface pressure (Figure 1A), but the effect was not dramatic with total insertion into TBLE of $18.2 \pm 1.3\%$, TBLE with 7 wt % cholesterol of $16.1 \pm 1.1\%$, and TBLE with 16 wt % cholesterol of $15.4 \pm 1.0\%$. Of note in all cases, regardless of the amount of exogenous cholesterol, the peptide inserted

into the lipid monolayer at a surface pressure higher than the ESP indicating a driving force for the peptide to interact with the membrane.

To understand why the addition of cholesterol to TBLE membranes would affect htt peptide insertion, individual surface pressure versus mean molecular area isotherms were measured for a series of binary mixtures of TBLE and cholesterol and additivity plots constructed (Supplemental Figure 1).⁵⁸ Comparison of the mean molecular area of the binary mixtures to that of a theoretical mean area consistent with ideal mixing of the two components provides evidence that exogenous cholesterol causes condensation or a stiffening of the monolayer (Supplemental Figure 1B) which may affect the ability of the htt peptide to insert into the layer.

Cholesterol Reduces the Susceptibility of Vesicles to htt-Induced Permeability

It has been previously shown that Nt¹⁷-Q₃₅-P₁₀-KK peptides can permeabilize TBLE bilayer lipid membranes.²⁵ To determine if additional cholesterol altered the susceptibility of membranes to permeabilization, calcein dye leakage assays with LUVs were performed. Disruption of the membrane by an external agent, via mechanisms ranging from pore formation to complete vesicle lysis, results in calcein release, dilution, and fluorescence emission from the unquenched dye; this allows permeabilization to be correlated with fluorescence increases. Solutions of predominately monomeric peptides were mixed with vesicles comprised of TBLE containing varying amounts of exogenously added cholesterol. Several lipid:peptide ratios ([peptide] = 7.34–470 nM) were used and fluorescence measured as a function of time, though a signal maximum was typically reached within several minutes. Nt¹⁷-Q₃₅-P₁₀-KK was able to induce significant calcein release from LUVs comprised of pure TBLE compared to control volumes of TFA/PBS buffer (Figure 1B). As the cholesterol content of the LUVs was increased, the extent of calcein dye leakage induced by exposure to Nt¹⁷-Q₃₅-P₁₀-KK drastically decreased, with minimal leakage at the highest cholesterol levels. The addition of just 2.3 wt % cholesterol to TBLE LUVs reduced permeabilization by more than half at all but the highest peptide concentrations. Reduced susceptibility of cholesterol containing LUVs to Nt¹⁷-Q₃₅-P₁₀-KK induced permeabilization was surprising as the peptide still had an appreciable affinity for monolayers containing up to 16% exogenous cholesterol as measured by Langmuir isotherms. This suggests that the peptides are still inserting into the membrane, but not causing a similar amount of disruption leading to content leakage.

Enriching TBLE with Cholesterol Alters the Extent of htt-exon1 Binding to Lipid Vesicles As Determined by a TBLE/PDA Binding Assay

To verify that altering the lipid content of TBLE would modulate the interaction of full-length htt-exon1 with lipids, we performed a TBLE/PDA vesicle lipid binding assay (Figure 2). This is a colorimetric assay in which mixed vesicles composed of phospholipids and polymerized PDA lipids exhibit rapid visible color changes from blue to red induced by interactions between peptides and the phospholipid moieties within the vesicles. The colorimetric response can be used to quantify peptide–membrane interactions and interfacial membrane processes. The TBLE/PDA vesicles were systematically enriched with 0% (pure TBLE), 10%, 20%, or 30% cholesterol by mass. These vesicles were exposed to 20 μ M htt-

exon1 (51Q) and %CR was measured for 16 h (Figure 2). Typically, there is an initial steady increase in %CR as TBLE/PDA vesicles are exposed to htt-exon1(51Q). With time, the % CR tends to level off to a quasi-steady state. As the exogenous cholesterol content was enriched from 0% to 30%, the binding of htt-exon1(51Q) to the vesicles was reduced. That is, the initial increase in %CR was less steep with increasing amounts of cholesterol. The quasi-steady state %CR is also significantly smaller in a cholesterol dose-dependent manner, indicating that cholesterol reduces htt-exon1(51Q) overall binding to and insertion into lipid membranes. Of note, there is still an appreciable interaction of htt-exon1(51Q) with the lipid vesicles at high exogenous cholesterol levels which parallels results seen in the htt peptide monolayer insertion measurements.

Cholesterol Modulates the Morphological and Mechanical Changes in Lipid Bilayers Associated with Exposure to htt

To investigate the influence of cholesterol on the aggregation of htt on lipid membranes, supported TBLE bilayers containing various amounts of cholesterol were exposed to htt-exon1(51Q) and continuously imaged using in situ AFM, allowing for the direct tracking of protein aggregation on the bilayer. Supported lipid bilayers retain several properties, such as lateral fluidity, associated with free membranes,⁵⁹ and have been used as model surfaces to study the interaction of several amyloid-forming proteins with lipid membranes.^{60–65}

Bilayers comprised of pure TBLE, TBLE + 10% cholesterol, TBLE + 20% cholesterol, and TBLE + 30% cholesterol were used. First, it was confirmed that continuous supported bilayers of the different lipid mixtures would form via vesicle fusion and remain structurally stable, i.e., no observed morphological changes, when imaged for several hours (Supplemental Figure 2). Only continuous TBLE bilayers ($40 \times 40 \mu\text{m}$ in size), as assessed by AFM imaging, were exposed to htt-exon1(51Q), and observations were limited to these verified continuous bilayers. After formation of an appropriate supported bilayer, the fluid cell was washed with neat buffer to reduce the amount of lipid components not bound to the mica substrate. Pure TBLE bilayers and those with exogenously added cholesterol were all smooth in appearance (RMS roughness less than 0.2 nm) with no indication of distinct lipid domains in either AFM height or phase images (Supplemental Figure 2).

Having established that supported bilayers appropriate for this study could be made from the different lipid mixtures, these model bilayers were systematically exposed to freshly prepared aliquots of htt-exon1(51Q) at a final protein concentration of $20 \mu\text{M}$ in the fluid cell. When pure TBLE bilayers were exposed to $20 \mu\text{M}$ solutions of htt exon1(51Q), extensive areas of the bilayer displayed disrupted morphology characterized by increased surface roughness and the accumulation of discrete htt aggregates within 10 min. These regions increased in size and became steadily rougher as a function of time (Figure 3 and Supplemental Movie 1). The disrupted regions appeared to be mechanically altered from the unperturbed regions of the bilayer, as there is distinct contrast observed in AFM phase images. This is consistent with previous reports that correlated the morphological changes associated with exposure of TBLE bilayers to htt-exon1(51Q) with decreased compression modulus of the membrane.^{25,28} During the 120 min of observation, the phase contrast

between the different regions remains consistent, suggesting that differences in elasticity between these distinct regions persist.

Aggregates observed on the pure TBLE bilayer appeared globular in nature (examples indicated by blue arrows in Figure 3). These aggregates were often stable, appearing in subsequent images of the same region; however, they did increase in volume as a function of time. In contrast to incubation experiments that demonstrate that htt-exon1(51Q) forms fibrils in the absence of lipids within an hour and increase in number thereafter (Supplemental Figure 3),⁶⁶ fibrils were not observed on lipid bilayers during the time course of the experiment. This suggests that the pure TBLE bilayer either promotes the formation of aggregates off pathway to fibrils or that aggregate intermediates are stabilized by interaction with the membrane.

When TBLE + 10% cholesterol bilayers were exposed to 20 μ M htt-exon1(51Q), morphological changes in the bilayer were observed that evolved with time (Figure 4 and Supplemental Movie 2). As the development of these domains could be directly tracked by continuous AFM imaging, these morphological changes arose from direct interaction of htt-exon1(51Q) with the bilayer. Initially, small domains of slightly increased height were detected in the TBLE + 10% cholesterol bilayers, and these domains were associated with clear contrast in AFM phase images, suggesting that these regions differed in their elastic properties compared to the regions of unaffected bilayer. With time, these domains grew in size and increased in height. The increase in height was correlated with a loss of contrast in corresponding phase images, indicating that these thicker domains had similar elastic properties compared with unperturbed bilayer. While a few globular aggregates were observed on the TBLE + 10% cholesterol bilayer, they were relatively rare compared to those that formed on the pure TBLE bilayers

The domains of altered morphology that developed in TBLE + 10% cholesterol bilayers were distinct compared to changes associated with exposure of pure TBLE bilayers to htt-exon1(51Q) (Figures 3B and 4B). While the disrupted regions that appeared in the pure TBLE bilayers were grainy in appearance (Figure 3B), the domains that developed in the TBLE + 10% cholesterol bilayers were plateau-like with a smoother appearance (Figure 4B). Both types of altered morphologies increased in height as presumably more material accumulated in these regions; however, the height increase was smaller in pure TBLE bilayers except when there was a discrete, globular aggregate. The smoother domains that developed on the TBLE + 10% cholesterol gradually grew from being ~1 nm to ~4 nm above the regions of unperturbed bilayer. In the absence of htt, these domains in TBLE bilayers with additional cholesterol did not develop.

When the amount of exogenously added cholesterol in the TBLE bilayers was increased to +20% or +30%, changes in membrane morphology similar to those associated with the +10% cholesterol bilayer were observed (Supplemental Figure 4). Specifically, small domains of slightly altered height and clear phase contrast were detected at initial time points. With time, these domains grew and transitioned toward the smooth, plateau-like morphology. However, as the cholesterol content was increased, the time required for this transition in morphology was extended. Furthermore, surface area affected by exposure to

htt-exon1(51Q) was reduced, consistent with the reduced insertion and binding of htt to lipid membranes via Langmuir and vesicle binding assays. The initial sharp phase contrast that dissipated with the development of these domains was also observed.

To quantify the interaction of htt-exon1(51Q) with the supported bilayers containing various amounts of cholesterol, the percentage of the surface that displayed any altered morphology was determined as a function of time via AFM image analysis (Figure 5). The extent of bilayer disruption consistently decreased as a function of increasing cholesterol content. This result is consistent with the TBLE/PDA lipid binding assay (compare with the highlighted region in Figure 2), as it also demonstrated a significant decrease in the interaction between htt-exon1(51Q) and the lipid vesicles within the first 2 h of the assay.

DISCUSSION

In this study, we investigated the role of cholesterol in modulating the binding of model htt peptides (both synthetic and recombinant) to lipid membranes and the associated consequences. Importantly, these two peptide systems have been shown to interact similarly with lipid bilayers.²⁵ Here, we report several findings. We demonstrate that increased cholesterol content reduces the insertion and binding of N-terminal htt fragments to lipid membranes. While there is still appreciable binding of htt-exon1 to membranes with up to 30% exogenously added cholesterol, there is a stark decrease in the ability of Nt¹⁷-Q₃₅-P₁₀-KK peptides to permeabilize lipid vesicles at additions as small as 2.3 wt % cholesterol. Furthermore, the morphological change in TBLE bilayers associated with exposure to htt-exon1 are drastically altered upon the addition of cholesterol. Pure TBLE bilayers develop regions of grainy, disrupted morphology that contain numerous globular aggregates. These regions are associated with stark contrast in AFM phase images which is indicative of altered elastic properties. However, the morphological changes induced in bilayers by exposure to htt-exon1 are drastically altered with the addition of cholesterol. Under these circumstances, discrete, thicker domains develop on the surface, and relatively few globular htt-exon1 aggregates are observed. Furthermore, the extent of bilayer that exhibits altered morphology as a result of exposure to htt-exon1 is significantly reduced as cholesterol content increases.

While it is clear that cholesterol homeostasis is altered in HD, the exact impact on cholesterol levels in the brain in HD remains controversial, as conflicting reports are present in the literature. In clonal striatal-derived cells that overexpress htt with expanded polyQ domains, mRNA levels of important genes associated with cholesterol biosynthesis appear to be reduced.⁶⁷ This has also been observed in brain HD mice and in post-mortem HD cerebral specimens.⁴¹ Reduced levels of cholesterol precursors were detected, via a variety of biochemical and mass spectrometry assays, during the initial stages of disease in brains from transgenic mouse and rat models of HD, and decreased levels of sterols/cholesterol were observed in these same models as disease progressed.^{41–43} Alterations in cholesterol homeostasis also appear in early stages of HD in humans.^{68,69} However, there are also conflicting reports that cholesterol accumulation occurs in mouse and human HD tissues and cell cultures.^{45–47} These reports suggest that htt with an expanded polyQ domain interacts with caveolin-1, resulting in the accumulation of intracellular cholesterol in primary neurons

and in brains from YAC72 transgenic mice.⁴⁵ The accumulation of cholesterol in HD cellular and animal models, as well as in HD human brains, contributed to NMDA-mediated excitotoxicity.⁴⁷ It has been suggested that limitations on the assays used to quantify cholesterol levels in these different studies may be responsible for the diverging results.⁷⁰ Despite these conflicting reports on the fate of cholesterol in HD, our results clearly demonstrate that the presence of cholesterol has pronounced effects on htt aggregation and its ability to bind and permeabilize lipid membranes. Our observations either illustrate a mechanism through which reduced cholesterol levels in neurons contribute to the misfolding of mutant htt leading to subsequent membrane perturbation or alternatively show how increased cholesterol levels in HD brains could represent a means by which neurons are protected from disruption induced by misfolded htt. This further justifies the need to resolve the controversy regarding altered cholesterol homeostasis in HD.

An interesting observation in cells expressing mutant htt was an increased number of membrane rafts associated with accumulation of cholesterol.⁴⁷ Here, we observed the development of large domains that were reminiscent of raft domains in bilayers that had been enriched with cholesterol and exposed to htt-exon1(51Q). These domains were, however, much thicker than is normally associated with lipid raft domains observed in pure lipid bilayers,^{71,72} but this may be partially due to the incorporation of the htt protein into these domains. Presumably, the formation of these domains was facilitated by the presence of htt-exon1(51Q) and the excess levels of cholesterol contained within the bilayer; therefore, it is reasonable to speculate that these domains are enriched in both htt and cholesterol. Lipid/htt interactions are also relevant in the htt trafficking within the cell,^{9,73,74} and cholesterol appears to mediate the affinity of htt for lipid surfaces.

There appears to be a subtle balance between the initial affinity of htt-exon1(51Q) to bind or insert into the lipid bilayer, which appears to be impeded by increasing cholesterol content, and the availability of cholesterol to aid in forming these domains once htt-exon1(51Q) has inserted into the bilayer. Both Nt¹⁷-Q₃₅-P₁₀-KK and htt-exon1(51Q) binding and insertion is impeded by the condensation or a stiffening effect of adding cholesterol to the membrane. Furthermore, the htt-exon1 that does insert into cholesterol enriched membranes behaves very differently, in how it alters the bilayer morphology and aggregates. Importantly, the development of these domains does not appear to be associated with membrane leakage induced by exposure to htt. However, it should be noted that the leakage assays were performed with Nt¹⁷-Q₃₅-P₁₀-KK, and the AFM studies were performed with htt-exon1(51Q). Another potential scenario by which cholesterol reduces the affinity of htt for lipid membranes is by restricting the efficient transition of the Nt¹⁷ into an amphipathic α -helix, a structure which is associated with its ability to bind lipids.^{25-27,75} Hydrogen bonding with phospholipid headgroups appears to stabilize α -helix formation in Nt¹⁷, facilitating binding to lipid membranes.⁷⁶ It is possible that additional cholesterol content may interfere with the formation of these important hydrogen bonds.

It has been hypothesized that the interaction of htt with membranous surfaces may mediate pathogenesis in a numerous ways, including stabilization or promotion of specific aggregate species, altered mechanical integrity of membranes leading to their dysfunction, and in trafficking of htt within the cell. The polyQ repeat in htt adopts a variety of conformations as

a monomer⁷⁷ and in aggregated species.⁷⁸ It is plausible that a subset of these conformers mediate pathogenesis in HD, and in this regard, the monoclonal antibody 3B5H10 recognizes a species of polyQ that strongly predicts neuronal death in cell culture.⁷⁹ If specific htt conformations play an important role in HD, understanding environmental factors that promote the relative abundance of disease relevant conformations may provide key insights into mechanisms associated with htt aggregation and toxicity. Lipid membranes represent such an environmental factor and have already been found to promote the formation of distinct aggregate species of htt fragments depending on the flanking sequences surrounding the polyQ domain.²⁵ Here, we demonstrated that the accumulation and/or aggregation of htt-exon1 with an expanded polyQ domain can be further modulated by changing the cholesterol content in lipid bilayers. Computational studies have demonstrated that when a variety of model htt peptides directly interact with DOPC membranes that there is an increase in the β -sheet content within the peptide.⁷⁶ Because of the lack of distinct htt aggregates observed on lipid bilayers enriched in cholesterol, it may be that cholesterol interferes with the ability of htt to transition to β -sheet rich structure that promotes aggregation.

The ability of protein aggregation to disrupt membrane integrity has been proposed as a potential mechanism associated with several neurodegenerative diseases.⁸⁰ In this regard, the impact of htt on membrane integrity and mechanics was highly dependent on cholesterol content. Mechanical changes to lipid bilayers induced by htt exon1 could underlie membrane dysfunction observed in animal models,^{6,81} and it appears that increased levels of cholesterol may protect against these changes. However, it should be noted that, due to experimental convenience, the levels of exogenously added cholesterol used in this study were larger than would be typically observed in a cellular membrane. The development of globular htt aggregates on the surface was highly correlated with the development of regions of increased bilayer roughness observed upon exposure of pure TBLE bilayers to htt-exon1(51Q), while addition of cholesterol ameliorated these specific effects. Additionally, pure TBLE vesicles, when exposed to Nt¹⁷-Q₃₅-P₁₀-KK, had markedly higher permeability compared to those with exogenous cholesterol. This suggests that the formation of these globular htt aggregates may play a mechanistic role in htt-induced membrane permeabilization.

In the interaction of htt with lipid membranes, cholesterol appears to play a significant role. The affinity of htt for membranes decreased with increasing amounts of exogenously added cholesterol, and the addition of cholesterol significantly reduced the ability of htt to damage membranes. The reduced ability to damage membranes enriched in cholesterol may be attributed to the manner in which htt alters membrane morphology and aggregates on the membrane surface. Collectively, these observations suggest that alterations in cholesterol homeostasis associated with HD may play an important role in dictating the ability of htt to aggregate into specific species and damage cellular membranes. While the studies presented here demonstrate that cholesterol concentration has a dramatic effect on htt binding to and aggregation on lipid membranes, further structural studies may clarify the mechanisms underlying these observations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AFM	atomic force microscopy
chol	cholesterol
HD	Huntington disease
HFIP	hexafluoroisopropanol
htt	huntingtin
GST	glutathione S-transferase
LPLC	low pressure liquid chromatography
LUVs	large unilamellar vesicles
%CR	% colorimetric response
PBS	phosphate-buffered saline
PDA	polydiacetylene
polyQ	polyglutamine
polyP	polyproline
Nt¹⁷	the first 17 amino acids of htt
TBLE	total brain lipid extract
TFA	trifluoroacetic acid

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Figure 1.

(A) Insertion of Nt¹⁷-Q₃₅-P₁₀-KK into lipid monolayers of varying TBLE:cholesterol ratios; expressed as % area increase at each surface pressure. (B) Calcein leakage, measured as relative fluorescence, from LUVs composed of different TBLE:cholesterol ratios exposed to Nt¹⁷-Q₃₅-P₁₀-KK (or buffer acting as a control) as a function of peptide:lipid ratio. Error bars indicate one standard deviation ($n = 3$).

Figure 2.

Percent colorimetric response (%CR) of TBLE/PDA vesicles containing various amounts of cholesterol upon exposure to htt-exon1(51Q) plotted as a function of time. Error bars indicate one standard deviation ($n = 3$). The highlighted area indicates the time frame accessible with AFM experiments described later.

Figure 3.

(A) Sequential AFM height and phase images taken in solution of supported TBLE bilayers exposed to htt-exon1(51Q). Blue arrows indicate oligomer-like structures. (B) AFM height images taken as a function of time demonstrating the rough, grainy morphological changes induced in a pure TBLE bilayer by htt-exon1(51Q). Blue lines correspond to the height profile presented directly below the images.

Figure 4.

(A) Sequential AFM height and phase images taken in solution of supported TBLE bilayers containing 10% exogenously added cholesterol exposed to htt-exon1(51Q). (B) AFM height images taken as a function of time demonstrating the smooth morphological changes induced in a TBLE + 10% cholesterol bilayer by htt-exon1(51Q). Blue lines correspond to the height profile presented directly below the images.

Figure 5. Percent area of disrupted bilayer morphology associated with exposure of lipid bilayers containing various amounts of cholesterol to htt-exon1(51Q) presented as a function of time. Error bars indicate one standard deviation ($n = 3$).