Biochemical and Biophysical Research Communications 387 (2009) 472–475

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Polyglutamine expansion in huntingtin increases its insertion into lipid bilayers

Kimberly B. Kegel [a,*], Vitali Schewkunow [b], Ellen Sapp [a], Nicholas Masso [a], Erich E. Wanker [c], Marian DiFiglia [a], Wolfgang H. Goldmann [b]

[a] Department of Neurology, Massachusetts General Hospital, Charlestown, MA 02129, USA
[b] Zentrum für Medizinische Physik und Technik, Biophysik, Universität Nürnberg-Erlangen, 91052 Erlangen, Germany
[c] Abt. Neuroproteomics, Max Delbrück-Zentrum für Molekulare Medizin (MDC) Berlin-Buch, 13125 Berlin, Germany

ARTICLE INFO

Article history:
Received 2 July 2009
Available online 14 July 2009

Keywords:
Differential scanning calorimetry
Huntingtin
Huntington disease
Membrane
Phosphatidylethanolamine
Polyglutamine

ABSTRACT

An expanded polyglutamine (Q) tract (>37Q) in huntingtin (htt) causes Huntington disease. Htt associates with membranes and polyglutamine expansion in htt may alter membrane function in Huntington disease through a mechanism that is not known. Here we used differential scanning calorimetry to examine the effects of polyQ expansion in htt on its insertion into lipid bilayers. We prepared synthetic lipid vesicles composed of phosphatidylcholine and phosphatidylethanolamine and tested interactions of htt amino acids 1–89 with 20Q, 32Q or 53Q with the vesicles. GST-htt1–89 with 53Q inserted into synthetic lipid vesicles significantly more than GST-htt1–89 with 20Q or 32Q. We speculate that by inserting more into cell membranes, mutant huntingtin could increase disorder within the lipid bilayer and thereby disturb cellular membrane function.

Introduction

Huntington disease (HD) is caused by an expanded CAG repeat in the HD gene which encodes the protein huntingtin (htt). The CAG repeat is translated into an expanded glutamine (Q) repeat near the N-terminus of htt, a large protein comprised of 3144 amino acids. Htt is a soluble protein that is normally found in association with membranes in cells [1–3] and in brain [4,5]. We found that htt with a polyglutamine stretch in the normal range (<37Q) associates *in vitro* with large unilamellar vesicles (LUVs) containing specific phospholipids including: PI(3,4)P2, PI(3,5)P2, PI(3,4,5)P3 and cardiolipin [6]. A cationic membrane-binding domain of amino acids 171–372 is necessary for htt-membrane association in cells [7]. However, htt residues 1–18 and 1–89 (exon1) fused to GFP or GST respectively, can also target to membranes [8,9], and htt1–102 can interact with phospholipids, although much less than longer htt fragments containing htt amino acids 171–372 [6].

Htt interactions with membrane phospholipids are polyglutamine-dependent. For instance, compared to wild-type full-length htt with 7Q, mutant htt with 140Q had increased association with vesicles enriched in phosphatidylethanolamine (PE) as measured by binding to LUVs followed by sedimentation though sucrose gradients [6]. *In vitro* translated mutant htt1–102 with 100Q interacts more with lipid vesicles compared to its wild-type counterpart with 18Q. These data suggest that polyglutamine expansion in htt increases its membrane phospholipids association and that a region within amino acids 1–102 of htt is responsible for the interactions.

Pure polyglutamine tracts can form channels in planar lipid bilayers [10,11], indicating that polyglutamine tracts alone could damage membranes. A key question is whether polyglutamine expansion in the context of htt can alter htt membrane insertion. An increase in membrane insertion with expanded polyglutamine would provide support for a direct effect on membrane stability as a potential pathogenic mechanism in HD. Previous work using a dye release assay showed that htt1–89 could disrupt lipid bilayers but no change with polyglutamine length was found [9]. Differential scanning calorimetry (DSC) is a much more sensitive method for detecting membrane disturbances since it can detect small changes in the physical properties of lipid bilayers caused by interactions with the membrane, whereas dye release assays require outright loss of membrane integrity. In this study, we used DSC

Abbreviations: DCS, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; EPA, ethyl-eicosapentaenoic acid; GABA, γ-aminobutyric acid; HD, Huntington disease; htt, huntingtin; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI(3,4)P2, phosphatidylinositol-3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol-3,5-bisphosphate; PI(3,4,5)P2, phosphatidylinositol-3,4,5-trisphosphate; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-ethanolamine; PS, phosphatidylserine; Q, glutamine.

* Corresponding author. Address: Department of Neurology, Massachusetts General Hospital East, 114 16th Street, Room 2125, Charlestown, MA 02129, USA. Fax: +1 617 726 1264.
E-mail address: kkegel@partners.org (K.B. Kegel).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.07.039

to study the effects of polyglutamine expansion near the N-terminus of htt on membrane insertion into PE containing vesicles. Results showed that polyglutamine expansion increased penetration of GST-htt1–89 into the lipid bilayer. These data support htt interactions with membranes through direct phospholipid association and membrane insertion. Through enhanced phospholipid interaction and membrane insertion, mutant htt may change normal membrane function.

Materials and methods

Western blots and silver staining. Proteins were separated by SDS–PAGE using 4–12% bis–tris gels (Invitrogen); proteins were transferred to nitrocellulose using iBlot semi-dry transfer apparatus (Invitrogen) and developed using chemiluminescence or gels were silver-stained using the Silver Stain Plus kit (BioRad). Blots were probed with Ab1, an antibody to the N-terminus of htt (htt1–17) [4].

Preparation of GST-htt fusion proteins. GST-htt exon1 with 18Q, 32Q, or 53Q were expressed in *Escherichia coli* BL21 Gold (Stratagene, La Jolla, CA) grown in 2YT broth and induced with isopropyl 1-thio-β-D-galactopyranoside. Proteins were purified and prepared as previously described [7] and purity was verified by Coomassie staining (BioSafe Coomassie G250 stain, BioRad) following SDS–PAGE.

Differential scanning calorimetry. The phospholipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids and used without further purification. Lipid stock solutions were prepared by dissolving the lipid powders in chloroform/methanol 2/1 (v/v). From aliquots of these solutions, a dry lipid film was formed on the walls of an extensively rinsed glass beaker by evaporating the solvent with a stream of nitrogen followed by vacuum desiccation for at least 3 h. The lipid film was dissolved in 15 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.5 for the preparation of multilamellar vesicles (MLVs). The lipid dispersion was then pressed 10 times through 200-nm filters to make LUVs and used in experiments at these buffer conditions [12]. A differential scanning calorimeter Q100 from TA Instruments was used. Briefly, the reservoirs for the sample and reference solution are made of stainless steel to hold a volume of around 100 μl each. Lipid vesicles solutions are placed in the reference cell and the lipid vesicle solutions plus the huntingtin constructs in the sample cell. Under sealed conditions, both solutions are heated/cooled at a rate of 0.5 °C/min between +7 °C and +35 °C in six to eight cycles until the equilibrium of the phase transition enthalpy was reached, using a mixture of DMPC:POPE (50:50 M ratio) at 5 mg/ml. A phase transition was observed at around 20 °C. Traces from various cycles (at different protein/lipid molar ratios) were averaged and data analysis was performed using the software from *Universal Analysis 2000* (TA Instruments) and *Origin 7G* [13].

Results

Protein–phospholipid interactions can modify membrane fluidity [14]. Altered fluidity of lipid bilayers can be measured by variations in the thermodynamic behavior of the membrane, which is determined by the amount of heat required to bring about a phase transition of the lipid bilayer. Protein–lipid interactions also change the melting temperature (T_m), which is otherwise a fixed value intrinsic to the lipid composition of the membrane. Having observed wild-type and mutant htt associated with synthetic lipids vesicles [6], we asked if an N-terminal fragment bearing the polyglutamine region can change the membrane fluidity. We used DSC to examine membrane insertion of bacterially expressed and purified GST-htt1–89 with 20Q, 32Q or 53Q. GST-htt1–89 could be purified in sufficient quantities needed for DSC measurements. Purified proteins of the expected sizes of (~26 kDa for GST) and 35–45 kDa for GST-htt1–89 with 20Q, 32Q and 53Q were eluted from the glutathione beads (Fig. 1). Note, that the polyglutamine tract reduces mobility in SDS–PAGE beyond what is predicted. Additional low molecular weight bands were observed on the Coomassie gel for each of the GST-htt products (Fig. 1A). We verified by Western blot that the full-length protein was GST-htt and additional bands were proteolytic breakdown products of the expressed GST-htt fusion proteins (Fig. 1B) and that the band around 26 kDa was GST (not shown). We used LUVs with the lipid combination of DMPC:POPE (1:1 M ratio) that is suitable for DSC analysis. Thermal heating profiles (heat flow in mW) are shown in Fig. 2. The phase transition peak or melting temperature (T_m) for DMPC:POPE (1:1) was ~19.2 °C. The relative flattening of the curves together with a shift of T_m to lower temperatures at increasing protein concentration are indicative for protein insertion into lipid bilayers [15,16]. Results showed that, for LUVs composed of DMPC:POPE, all three GST-htt1–89 proteins reduced the enthalpy required to bring about a phase change compared to GST alone (Fig. 3). The reduced enthalpy requirement reflects insertion of GST-htt1–89 in the lipid bilayer, causing more disorder in the bilayer and a reduced energy requirement for melting. At 5 μM protein concentration, LUVs required significantly less heat for phase transition in the presence of GST-htt1–89Q32 compared to GST-htt1–89Q20, and GST-htt1–89Q53 compared to GST-htt1–89Q20 ($p < 0.0001$, $n = 4$–6, unpaired Student's t-test). At 10 μM protein concentration, GST-htt1–89Q53 was significantly different from both GST-htt1–89Q20 and GST-htt1–89Q32 ($p < 0.0001$, $n = 6$, unpaired Student's t-test). Protein concentrations higher than 10 μM (data not shown) gave variable results in the assay, possibly due to increased aggregation of htt during the reaction [9]. Due to the presence of proteolytic breakdown products in our preparation, we cannot say whether the insertion was due to the GST-htt1–89 fusion protein or due to free htt1–89 or smaller htt fragments generated through proteolysis. GST alone did not insert into LUVs. These results show that a region in htt1–89 can insert into a lipid bilayer in a polyglutamine-dependent manner.

Discussion

We used DCS to show that a small N-terminal fragment of htt inserts into vesicles composed of PC and PE, which are major

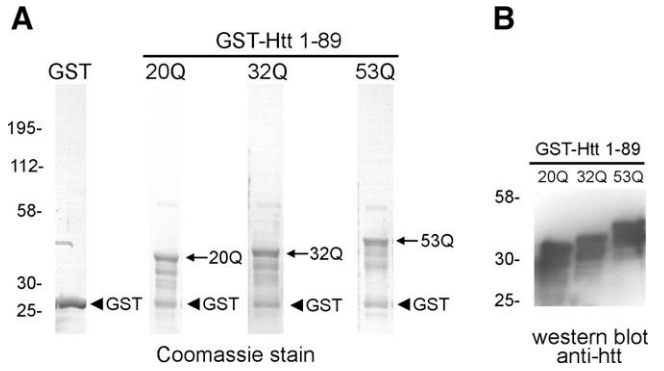

Fig. 1. Purity of GST-htt fusion proteins. (A) Coomassie stained gel of GST and GST-htt fusion proteins. GST runs at ~26 kDa. GST-htt1–89Q20, GST-htt1–89Q32 and GST-htt1–89Q53 run between 35 and 45 kDa (arrows). Due to proteolysis, GST alone was present in all preparations (arrowhead). Molecular mass is indicated in kDa on the *left*. (B) Western blot analysis was performed using anti-htt antibody Ab1. The lower molecular mass products (easily visible by Coomassie staining) were confirmed to be proteolytic products of htt by Western blot analysis.

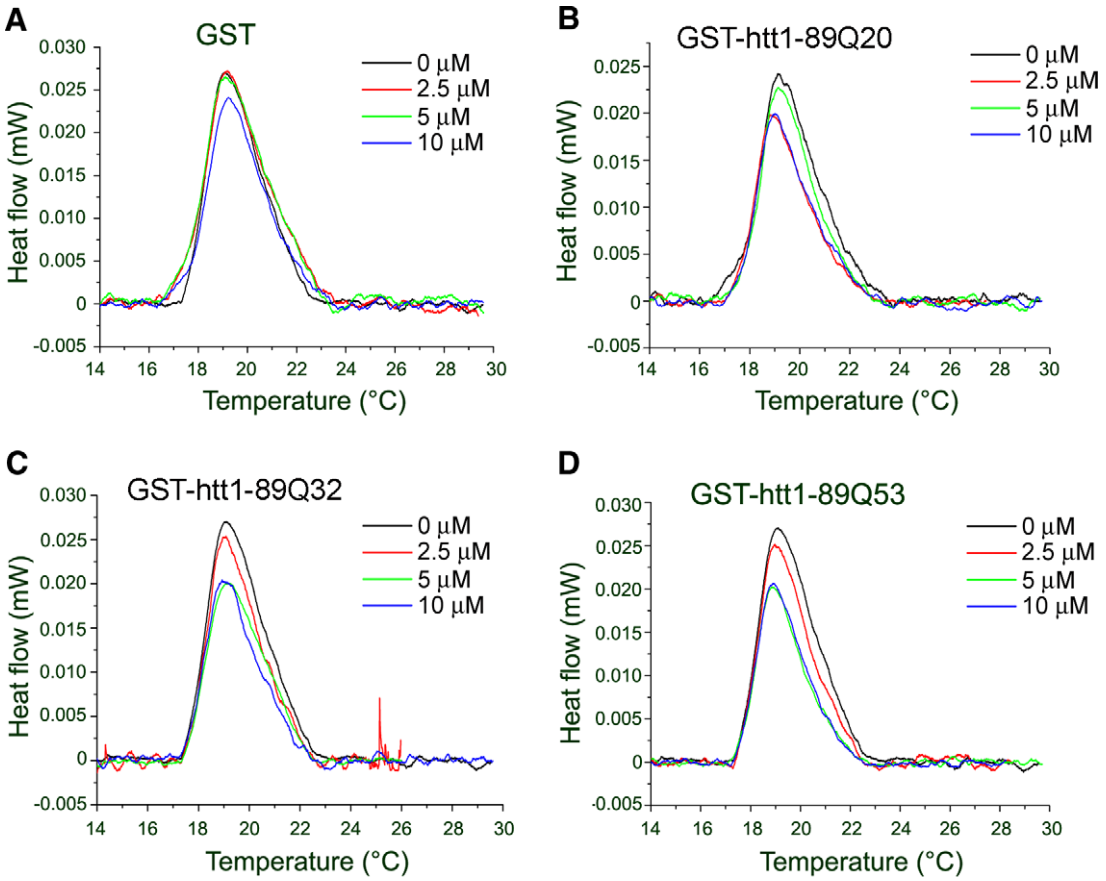

Fig. 2. Polyglutamine-dependent insertion of GST-htt1–89 measured using DSC. Htt interactions with LUVs. (A–D) Thermograms from DSC measurements with lipid vesicles containing DMPC and POPE at a 1:1 M ratio. The heat flow (mW) for pure GST (A) decreased minimally with increasing peptide concentration compared to GST-htt1–89Q20 (B), htt1–89Q32 (C), and htt1–89Q53 (D). (——) 0 μM or lipid only; (——) 2.5 μM; (——) 5 μM; (——) 10 μM protein (n = 4–6 runs).

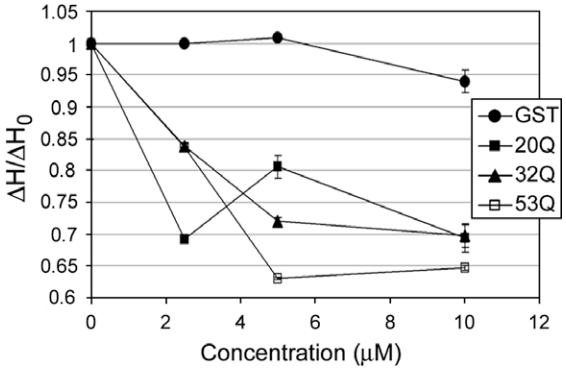

Fig. 3. Enthalpy changes ($\Delta H/\Delta H_o$) of PC/PE vesicles with different proteins (GST, and GST-htt1–89 with Q20, Q32 and 53Q). The relative enthalpy changes, ΔH normalized against ΔH_o (lipids only) were plotted at increasing protein concentration (0–10 μM). Most significant differences were observed between the huntingtin constructs at 5 μM, indicating lipid membrane insertion of the order GST-htt1–89Q53 > GST-htt1–89Q32 > GST-htt1–89Q20. GST used as control, showed little influence on enthalpy changes over the entire protein concentration range.

constituents of biological membranes. We found a decreased enthalpy (reduced ΔH) for phase transition observed with increasing polyglutamine length, indicating that polyglutamine expansion in htt increases its insertion into lipid bilayers. Previous studies using a dye release assay showed an association of htt1–89 with lipid vesicles that was not polyglutamine-dependent. Using a more sensitive method (DSC), we were able to demonstrate that polyglutamine expansion increases htt1–89 insertion into lipid bilayers.

Whether the increased insertion into model membranes by a purified mutant htt fragment is sufficient to explain membrane dysfunction *in vivo* is unknown. Early studies of membranes from control or HD brains suggested that the HD mutation could alter the physical properties of membranes. Membranes from HD brain samples showed increased binding affinities of tritium-labeled γ-aminobutyric acid (GABA) that were normalized by treatment with Triton X-100, phospholipase C, and glycerophospho-ethanolamine (a metabolite of PE) [17]. It is noteworthy that the addition of the polyunsaturated fatty acid ethyl-eicosapentaenoic acid (ethyl-EPA) to the diet reduces motor symptoms in a HD mouse model [18] and reduces motor symptoms and brain atrophy in HD patients [19,20]. The mechanism through which ethyl-EPA exerts its effect is unknown, but ethyl-EPA changes the physical properties of model membranes, including the melting temperature [21]. Thus, EPA may work to counteract adverse effects of mutant htt on the physical properties of biological membranes. A more dramatic effect on neuronal membranes would occur if mutant htt formed ionic conductance channels, which could dissipate membrane potentials across the plasma membrane and in mitochondria [22]. Pure polyglutamine tracts can form channels that accommodate physiological ions such as K^+, Na^+ and H^+ in planar lipid bilayers [22]. However, these studies did not provide direct evidence for an alteration in the lipid bilayer by mutant htt.

There is ample evidence for membrane dysfunction in a HD transgenic mouse expressing mutant htt1–89 (R62) with large polyglutamine repeats (>120Q). This mouse model develops a severe motor dysfunction and early death [23]. Levels of receptors important for normal synaptic transmission and membrane

conductance, including NMDARε, GluR1 and GluR2 were changed in post-synaptic membranes isolated from R6/2 mice [9]. Amphiphysin and synaptojanin are membrane-associated proteins important for vesicle trafficking of such receptors [24] and their levels were also changed in the same membrane preparations [9].

In summary, our findings using DSC suggest that mutant htt causes direct disturbance in the stability of the lipid bilayer and thereby could be a basis for the membrane dysfunction previously observed in brain of HD patients and R62 mice. Mutant htt could also indirectly affect membrane function by changing the physical properties of lipid bilayers and by altering binding of other soluble proteins. We propose that mutant htt interaction at membranes may represent a target for therapeutic intervention in HD.

Acknowledgments

We thank Ben Fabry for stimulating discussions. This work was funded by a Grant from CHDI to MD, Grants from the HDSA to M.D. and E.E.W, and by Grants from BFHZ, DAAD, BaCaTec to W.H.G.

References

[1] M.A. Kalchman, H.B. Koide, K. McCutcheon, R.K. Graham, K. Nichol, K. Nishiyama, P. Kazemi-Esfarjani, F.C. Lynn, C. Wellington, M. Metzler, Y.P. Goldberg, I. Kanazawa, R.D. Gietz, M.R. Hayden, HIP1, a human homologue of *S. cerevisiae* Sla2p, interacts with membrane-associated huntingtin in the brain, Nat. Genet. 16 (1997) 44–53.

[2] J. Velier, M. Kim, C. Schwarz, T.W. Kim, E. Sapp, K. Chase, N. Aronin, M. DiFiglia, Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways, Exp. Neurol. 152 (1998) 34–40.

[3] S. Waelter, E. Scherzinger, R. Hasenbank, E. Nordhoff, R. Lurz, H. Goehler, C. Gauss, K. Sathasivam, G.P. Bates, H. Lehrach, E.E. Wanker, The huntingtin interacting protein HIP1 is a clathrin and alpha-adaptin-binding protein involved in receptor-mediated endocytosis, Hum. Mol. Genet. 10 (2001) 1807–1817.

[4] M. DiFiglia, E. Sapp, K. Chase, C. Schwarz, A. Meloni, C. Young, E. Martin, J.P. Vonsattel, R. Carraway, S.A. Reeves, et al., Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons, Neuron 14 (1995) 1075–1081.

[5] A.H. Sharp, S.J. Loev, G. Schilling, S.H. Li, X.J. Li, J. Bao, M.V. Wagster, J.A. Kotzuk, J.P. Steiner, A. Lo, et al., Widespread expression of Huntington's disease gene (IT15) protein product, Neuron 14 (1995) 1065–1074.

[6] K.B. Kegel, E. Sapp, J. Alexander, A. Valencia, P. Reeves, X. Li, N. Masso, L. Sobin, N. Aronin, M. DiFiglia, Polyglutamine expansion in Huntingtin alters its interaction with phospholipids, J. Neurochem. (2009) Jun 29 [Epub ahead of print].

[7] K.B. Kegel, E. Sapp, J. Yoder, B. Cuiffo, L. Sobin, Y.J. Kim, Z.H. Qin, M.R. Hayden, N. Aronin, D.L. Scott, G. Isenberg, W.H. Goldmann, M. Difiglia, Huntingtin associates with acidic phospholipids at the plasma membrane, J. Biol. Chem. 280 (2005) 36464–36473.

[8] R.S. Atwal, J. Xia, D. Pinchev, J. Taylor, R.M. Epand, R. Truant, Huntingtin has a membrane association signal that can modulate huntingtin aggregation, nuclear entry and toxicity, Hum. Mol. Genet. 16 (2007) 2600–2615.

[9] J. Suopanki, C. Gotz, G. Lutsch, J. Schiller, P. Harjes, A. Herrmann, E.E. Wanker, Interaction of huntingtin fragments with brain membranes – clues to early dysfunction in Huntington's disease, J. Neurochem. 96 (2006) 870–884.

[10] Y. Hirakura, R. Azimov, R. Azimova, B.L. Kagan, Polyglutamine-induced ion channels: a possible mechanism for the neurotoxicity of Huntington and other CAG repeat diseases, J. Neurosci. Res. 60 (2000) 490–494.

[11] H. Monoi, S. Futaki, S. Kugimiya, H. Minakata, K. Yoshihara, Poly-L-glutamine forms cation channels: relevance to the pathogenesis of the polyglutamine diseases, Biophys. J. 78 (2000) 2892–2899.

[12] W.H. Goldmann, J.L. Niles, M.A. Arnaout, Interaction of purified human proteinase 3 (PR3) with reconstituted lipid bilayers, Eur. J. Biochem. 261 (1999) 155–162.

[13] W.H. Goldmann, B. Bechinger, T.P. Lele, Cytoskeletal proteins at the lipid membrane, in: Tien, Ottova-Leitmannova (Eds.), Advance in Planar Lipid Bilayers and Liposomes, Elsevier Inc., 2008, pp. 227–255 (Chapter 8).

[14] K.J. Seu, L.R. Cambrea, R.M. Everly, J.S. Hovis, Influence of lipid chemistry on membrane fluidity: tail and headgroup interactions, Biophys. J. 91 (2006) 3727–3735.

[15] M. Tempel, W.H. Goldmann, C. Dietrich, V. Niggli, T. Weber, E. Sackmann, G. Isenberg, Insertion of filamin into lipid membranes examined by calorimetry, the film balance technique, and lipid photolabeling, Biochemistry 33 (1994) 12565–12572.

[16] V. Schewkunow, K.P. Sharma, G. Diez, A.H. Klemm, P.C. Sharma, W.H. Goldmann, Thermodynamic evidence of non-muscle myosin II-lipid-membrane interaction, Biochem. Biophys. Res. Commun. 366 (2008) 500–505.

[17] K.G. Lloyd, L. Davidson, [^{3}H]GABA binding in brains from Huntington's chorea patients: altered regulation by phospholipids?, Science 205 (1979) 1147–1149

[18] J.J. Clifford, J. Drago, A.L. Natoli, J.Y. Wong, A. Kinsella, J.L. Waddington, K.S. Vaddadi, Essential fatty acids given from conception prevent topographies of motor deficit in a transgenic model of Huntington's disease, Neuroscience 109 (2002) 81–88.

[19] B.K. Puri, B.R. Leavitt, M.R. Hayden, C.A. Ross, A. Rosenblatt, J.T. Greenamyre, S. Hersch, K.S. Vaddadi, A. Sword, D.F. Horrobin, M. Manku, H. Murck, Ethyl-EPA in Huntington disease: a double-blind, randomized, placebo-controlled trial, Neurology 65 (2005) 286–292.

[20] B.K. Puri, G.M. Bydder, M.S. Manku, A. Clarke, A.D. Waldman, C.F. Beckmann, Reduction in cerebral atrophy associated with ethyl-eicosapentaenoic acid treatment in patients with Huntington's disease, J. Int. Med. Res. 36 (2008) 896–905.

[21] Y. Onuki, M. Morishita, Y. Chiba, S. Tokiwa, K. Takayama, Docosahexaenoic acid and eicosapentaenoic acid induce changes in the physical properties of a lipid bilayer model membrane, Chem. Pharm. Bull. (Tokyo) 54 (2006) 68–71.

[22] B.L. Kagan, Y. Hirakura, R. Azimov, R. Azimova, The channel hypothesis of Huntington's disease, Brain Res. Bull. 56 (2001) 281–284.

[23] S.W. Davies, M. Turmaine, B.A. Cozens, M. DiFiglia, A.H. Sharp, C.A. Ross, E. Scherzinger, E.E. Wanker, L. Mangiarini, G.P. Bates, Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation, Cell 90 (1997) 537–548.

[24] M.R. Wenk, P. De Camilli, Protein–lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals, Proc. Natl. Acad. Sci. USA 101 (2004) 8262–8269.