Original Article

DOI: 10.5582/bst.2012.v6.4.183

Membrane raft disruption results in neuritic retraction prior to neuronal death in cortical neurons

Shawn N. Whitehead[1], Sandhya Gangaraju[1], Amy Aylsworth[1], Sheng T. Hou[1,2,*]

[1] *Experimental Neurotherapeutics Laboratory, NRC-Institute for Biological Sciences, National Research Council Canada, Ottawa, Canada;*
[2] *Department of Biochemistry, Microbiology and Immunology, University of Ottawa,Ottawa, Canada.*

Summary **Membrane rafts, rich in sphingolipids and cholesterol, play an important role in neuronal membrane domain-specific signaling events, maintaining synapses and dendritic spines. The purpose of this study is to examine the neuronal response to membrane raft disruption. Membrane rafts of 8 DIV primary neuronal cultures were isolated based on the resistance to Triton X-100 and ability to float in sucrose gradients. Membrane rafts from primary cortical neurons were also imaged using the membrane raft marker, cholera toxin subunit-B (CTxB), and were co-immunolabelled with the dendritic microtubule associated protein marker, MAP-2, the dendritic and axonal microtubule protein, β-III-Tubulin, and the axonal microtubule protein, Tau. Exposure of cortical neurons to either the cholesterol depleting compound, methyl-beta-cyclodextrin (MBC), or to the glycosphingolipid metabolism inhibiting agent D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), resulted in neuritic retraction prior to the appearance of neuronal death. Further investigation into the effects of MBC revealed a pronounced perturbation of microtubule protein association with membrane rafts during neuritic retraction. Interestingly, stabilizing microtubules with Paclitaxel did not prevent MBC-induced neuritic retraction, suggesting that neuritic retraction occurred independently of microtubule disassembly and that microtubule association with membrane rafts is critical for maintaining neuritic stability. Overall, the data indicated that membrane rafts play an important role in neurite stability and neuronal viability.**

Keywords: Membrane raft, neuritic retraction, axonal damage, neuronal death, cortical neurons, time lapse imaging

1. Introduction

The plasma membrane of cells, composed mainly of lipids, is a fluid dynamic structure that has the ability to change its fatty acid composition and density to adjust and react to its external environment. One way to do this is through the formation of specialized membrane microdomains called lipid rafts, or membrane rafts, as the name has been redefined recently (*1*). These specialized membrane microdomains are rich in glycosphingolipids and cholesterol and are known to play an important role in neuronal domain specific signaling events (*2*) and maintaining synapses and dendritic spines (*3*). For example, membrane rafts compartmentalize cellular processes by serving as organizing centers or scaffolds for the assembly of axonal outgrowth signaling molecules (*4,5*), influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking (*3,6*). One key feature distinguishing membrane rafts from the rest of the plasma membranes is lipid composition. Membrane rafts generally contain twice the amount of cholesterol found in the surrounding lipid bilayer and have 50% more of sphingolipids, such as sphingomyelin, compared to the rest of the plasma membrane. Proteins in membrane rafts also undergo lipid modifications such as

**Address correspondence to:*
Dr. Sheng T. Hou, NRC-Institute for Biological Sciences, National Research Council Canada, 1200 Montreal Road, Bldg M54, Ottawa K1A 0R6, Canada.
E-mail: sheng.hou@nrc-cnrc.gc.ca

glycosylphosphatidylinositol anchors and acquire the ability to directly bind cholesterol (*7*).

The role of membrane rafts in maintaining axonal structure and neuritic networks in mature adult neurons is still unclear. Cholesterol rich membrane rafts have been shown to interact with components of the cytoskeleton (*8,9*). Specifically, α-tubulin has been co-immunoprecipitated with Caveolin-1, a marker for membrane rafts, in rat brain extracts (*10*). Also, membrane raft domains have been implicated in stabilizing microtubules within smooth muscle cells (*11*). These studies prompted us to hypothesize that disruption of membrane rafts through perturbing cholesterol may affect the cytoskeleton thereby causing morphological changes in neuritic structures and may ultimately affect membrane raft-mediated signal transduction and neuronal survival. Methyl-beta-cyclodextrin (MBC), a cyclic oligosaccharide with high a affinity to cholesterol, has been widely used to sequester cholesterol from cellular membranes and thus disrupt membrane rafts (*12*). Several studies have examined the effects of cholesterol depletion *via* MBC exposure on signal transduction pathways (*13-17*). D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) has also been shown to disrupt membrane raft integrity by inhibiting glycosphingolipid metabolism (*18-20*).

In the present study, the neuronal response to membrane raft disrupting agents MBC and D-PDMP were examined. Continued exposure of cortical neurons to either MBC or D-PDMP resulted in rapid neuritic retraction prior to the appearance of neuronal death. Several microtubule proteins were found to co-localize with membrane rafts indicating that membrane raft stability may be required for maintaining neuritic integrity. These findings shed light on the role of membrane rafts in modulating neuritic stability in mature cortical neurons.

2. Materials and Methods

2.1. *Culture of cerebral cortical neurons*

Primary cortical neurons were cultured from embryonic day 15/16 CD-1 mice as described previously (*21,22*). Briefly, neurons were plated into 24-well plates at a density of 1×10^6 cells/well coated with poly-D-lysine (100 μg/mL) in serum-free medium (N_2/Dulbecco's modified Eagle's medium (1:1) supplemented with 6 mg/mL D-glucose, 100 μg/mL transferrin, 25 μg/mL insulin, 20 nM progesterone, 60 μM putrescine, and 30 nM selenium. Neurons were incubated at 37°C with 5% CO_2. One-third of the medium was exchanged with fresh medium at 3 and 6 days *in vitro* (DIV).

2.2. *Induction of neuritic retraction*

Primary cortical neurons were treated with various concentrations (5 μM, 50 μM, 500 μM, 5 mM and 10 mM) of MBC (Sigma, Toronto, ON, Canada) diluted in ddH_2O for various time points up to 1 h. Neuritic retraction was also examined in primary neuronal cultures following exposure to various concentrations (1 μM, 5 μM, 10 μM and 20 μM) of D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (Sigma, Toronto, ON, Canada). To establish the effects of microtubule stability on MBC-induced neuritic retraction, primary cortical neurons were incubated with either dimethyl sulfoxide (DMSO) (Sigma, Toronto, ON, Canada) alone or with 100 nM Paclitaxel (Taxol) diluted in DMSO 2 h prior to exposure with MBC, a concentration and time point shown to be effective in protecting neurons against toxicity (*23*).

2.3. *Neuronal viability*

Neuronal viability was determined by an Alamar Blue (AB) assay (BioSource International, Nivelles, Belgium). This compound links to the respiratory chain without affecting the integrity of cells (*24*). When AB is reduced, it becomes fluorescent. Thus, cell metabolic rates can be evaluated proportionally with the fluorescence intensity. Briefly, at the end of the treatment period, 10% v/v AB was added to each well. Plates were then incubated at 37°C for 1 h. Next, for each well, three 100 μL aliquots were transferred to a 96-well plate and the absorbance at 540 nm was spectrophotometrically detected with a Cytofluor 2350 Fluorescent Measurement System. The absorbance of a medium blank with AB was subtracted from the absorbance of the control and test wells with AB to give the absorbance of the oxidized form and AB reduction was calculated. Each well was read in triplicate and experimental data was normalized to controls. All results represent the mean of two separate culture experiments. Neuronal viability was also calculated by detecting nuclei per microscopic field using Hoechst 33258 nuclear stain. Data was represented as percent viability and was calculated from five fields per treatment for two independent cultures.

2.4. *Labeling membrane rafts and fluorescent immunohistochemistry*

Coverslips containing 8 DIV primary cortical neurons were fixed with 4% formaldehyde (VWR, Mississauga, ON, Canada) in 10 mM PBS for 30 min at room temperature, followed by rinsing in 10 mM PBS for 5 times. For membrane raft labeling, coverslips were incubated with 5 μg of CTxB-FITC (Sigma, Toronto, ON, Canada) for 1 h on ice and covered to limit exposure to light. Coverslips to be used for

immunofluorecence were washed 3 times with PBS following CTxB incubation and incubated with the following primary antibodies in a buffer containing 10 mM PBS, 0.3% Triton X-100, 1.5% BSA for 1 h at room temperature: mouse Anti-β-III-tubulin monoclonal antibody (1:100, Millipore Laboratories, Billerica, MA, USA), mouse anti-MAP2a+b monoclonal antibody (1:500, Millipore Laboratories, USA) or mouse anti-Tau monoclonal antibody (1:100, Millipore Laboratories, Billerica, MA, USA). Coverslips were then washed 3 times in 10 mM PBS and were incubated in a donkey anti-mouse Cy-3 conjugated secondary antibody (1:400, Jackson laboratories, Bar Harbor, ME, USA). Following 3 washes in 10 mM PBS, coverslips were briefly dipped in water to prevent PBS crystal formation and mounted with Dako fluorescent mounting media (Sigma, Toronto, ON, Canada) spiked with 5 mg/mL Hoechst 33258 (Sigma, Toronto, ON, Canada). Coverslips containing labeled cells were visualized using a Zeiss inverted microscope with a magnification of 40× and digital images were obtained using the Axiovision v.4.7.2.0 software.

2.5. *Isolation of detergent resistant membranes*

Detergent-resistant membranes were isolated on the basis of their resistance to Triton X-100 at 4°C and their ability to float in sucrose density gradients. Primary cortical neurons were homogenized in 1 ml of PTN 50 buffer (50 mM sodium phosphate, pH 7.4; 1% Triton X-100, 50 mM NaCl) containing 10 mM DTT, 1 mM PMSF, 5 μg leupeptin and 1 μg pepstatin A. Cell lysates were centrifuged at 13,400 × g for 3 min to remove any nuclear material. The remaining supernatant was diluted with equal volumes of 80% sucrose. Samples were carefully overlaid with equal volumes of 30% and 5% sucrose respectively. The gradient was centrifuged at 130,000 × g (average) for 20 h at 4°C in a Beckman L8-70 ultracentrifuge (SW40-400 rotor) and aliquoted into 600 μL fractions. Following fractionation, fractions were collected and the protein concentrations within the fractions were detected using the Biorad protein assay (Biorad, Missisauga, ON, Canada). Membrane raft positive fractions were identified based on a positive western blotting signal with Caveolin-1 and Flotillin-1 (see western blotting for details). Membrane raft and non-raft fractions were then pooled and concentrated using Amicon ultra columns (Millipore Laboratories, Billerica, MA, USA).

2.6. *Western blotting*

Twenty microgramme of protein from each pooled fraction was separated by 6% or 8% SDS-PAGE. Electrophoresis was performed at 100 V and the gel was transferred to pure nitrocellulose membrane (Biorad laboratories, Mississauga, ON, Canada) for 1 h at room temperature at 100 V. The nitrocellulose membrane was blocked for 30 min at room temperature with 5% non-fat milk in TBST buffer containing 10 mM Tris, 150 mM NaCl, and 0.1% Tween-20. Membranes were incubated with the following primary antibodies overnight at 4°C: rabbit anti-Caveolin-1 polyclonal (1:500, Santa Cruz Laboratories, Santa Cruz, CA, USA), mouse anti-Flotillin-1 monoclonal (1:1,000, BD Biosciences, USA), anti-β-III-tubulin monoclonal (1:1,000, Millipore Laboratories, Billerica, MA, USA), mouse anti-MAP2a+b monoclonal (1:1,000, Millipore Laboratories, Billerica, MA, USA) or mouse anti-Tau monoclonal (1:500, Millipore Laboratories, Billerica, MA, USA). All primary antibodies were diluted in 5% non-fat milk. TBST: The primary antibody incubation was followed by 3 × 10 min washes in TBST followed by an incubation with the appropriate secondary antibodies diluted in 5% non-fat milk/TBST: HRP-conjugated secondary goat-anti-mouse (1:2,500, Jackson Laboratories, Bar Harbor, ME, USA) and HRP-conjugated secondary goat-anti-rabbit (1:2,500, Jackson Laboratories, Bar Harbor, ME, USA). Following 3 × 10 min washes in TBST, the blots were developed with ECL (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada).

2.7. *Time lapse imaging*

Primary cortical neurons were acclimated for 30 min to the time lapse chamber and were maintained at 37°C with 5% CO_2. Cells were then incubated with 30 μg/mL propidium iodide (PI) for 30 min. Following acclimation and incubation with PI, cortical neurons were exposed to 5 mM MBC. Cultures were imaged using time lapse microscopy imaging every 20 sec following MBC exposure for up to 1 h. During each time interval both phase and Cy-3 images were obtained.

2.8. *Data analysis*

All data was analyzed by one way ANOVA and further *post hoc* tests for significant groups using Dunnett's test. Groups were considered significant with a p-value < 0.05.

3. Results

3.1. *Exposure of cortical neurons to MBC or D-PDMP results in dose-dependent neuritic retraction and neuronal death*

To determine the effects of membrane raft disruption on neurons, a dose response curve of MBC was established. Primary cortical neurons at 8 DIV were exposed to 5 μM, 50 μM, 500 μM, 5 mM or 10 mM MBC for up to 30 min. At 20 and 30 min of exposure, cells were fixed. Neuritic length and neuronal viability

were assessed (Figure 1A, red colored lines). Based on Hoechst staining and nuclei morphology, a MBC exposure at 5 μM or 50 μM did not have any effect on neuronal viability (Figure 1A). Neuronal viability was significantly reduced by a 30 min exposure of 500 μM or 5 mM MBC. Neuronal viability was also significantly reduced when neurons were exposed to a higher dose of 10 mM MBC (data not shown). Given that membrane raft disruption from MBC appeared to result in a concentration dependant cell death, it is still possible that lower concentrations, (and earlier time points), may still impact neuronal structure and function. We therefore next examined the dose response of MBC exposure on neuritic retraction (Figure 1A, black colored lines). Neurons significantly retracted their neurites as early as 20 min when exposed to 50 μM, 500 μM, or 5 mM MBC. Therefore, these results demonstrated that exposure to MBC resulted in neuritic retraction followed by neuronal death.

Membrane raft disruption *via* inhibition of glycosphingolipid synthesis has also been documented using D-PDMP (*18-20*). Hoechst staining and nuclei morphology revealed that neurons treated with 10 μM D-PDMP started to die following 60 min of exposure

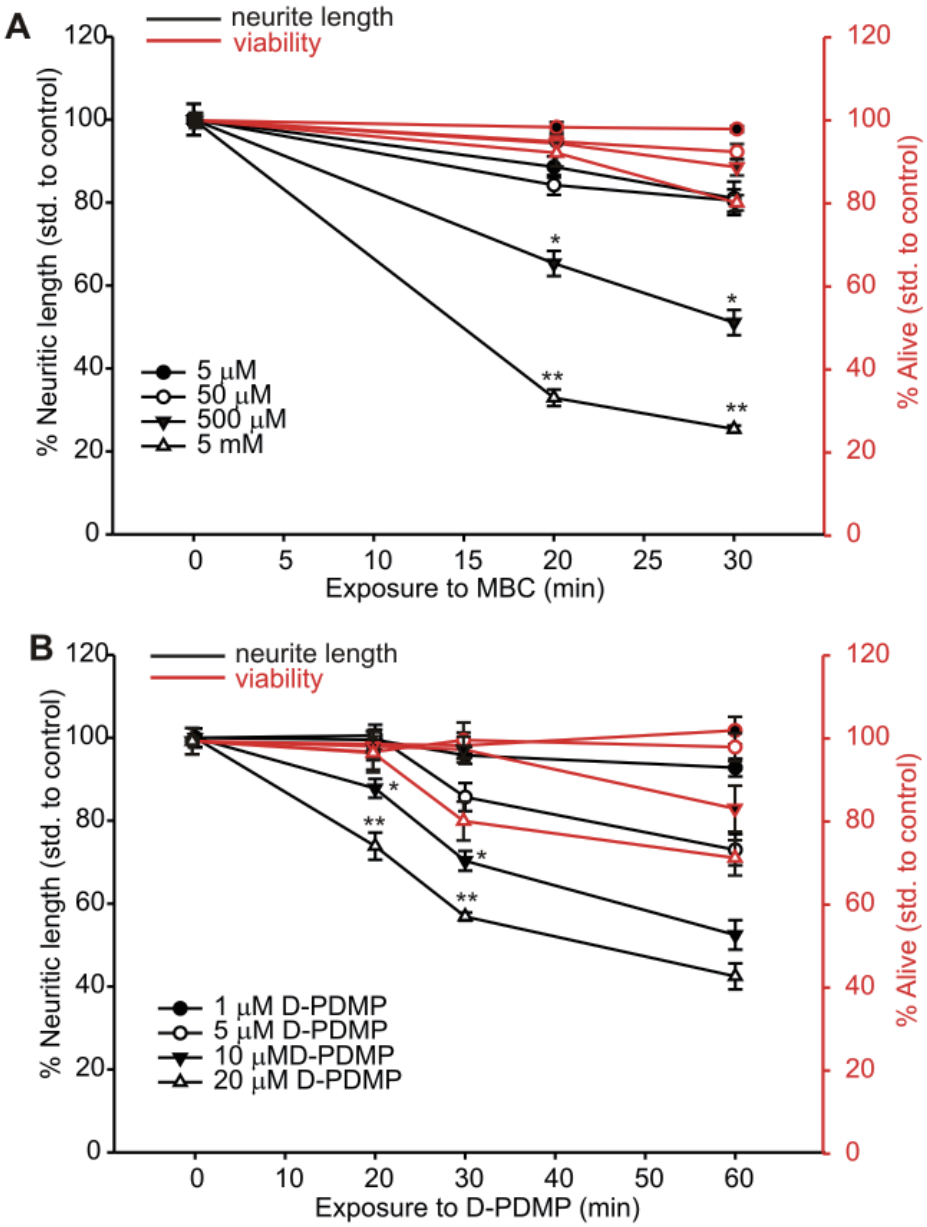

Figure 1. Effects of MBC and D-PDMP on neuritic length and neuronal viability. (A) Primary cortical neurons cultured for 8 DIV were exposed to 5 μM, 50 μM, 500 μM, 5 mM or 10 mM of MBC for 20 and 30 min. **(B)** Primary cortical neurons cultured for 8 DIV were exposed to 1 μM, 5 μM, 10 μM or 20 μM of D-PDMP for 20, 30 or 60 min. In both **(A)** and **(B)**, Neuronal viability (red colored lines) was determined on fixed cells stained with Hoechst 33258. Neuritic length (black colored lines) was measured and quantified using Image J software. Data from two independent experiments were presented as mean% alive from control untreated cells **(A** and **B)** and mean% change in neuritic length **(A** and **B)** ± S.E.M (* indicates statistical significant change at $p < 0.05$ from the control by one-way ANOVA with Dunnett's *post hoc* test, $n = 12$).

while 20 μM D-PDMP treated neurons resulted in cell death by 30 min of exposure (Figure 1B, red colored lines). This level of D-PDMP-induced toxicity was consistent with a previous study demonstrating the toxic effects of D-PDMP in cultured trigeminal sensory neurons whereby neurons died following exposure to 20 μM D-PDMP (*19*). Here, neurons significantly retracted their neurites by 30 min of exposure of 5 μM D-PDMP, and as early as 20 min when exposed to 10 μM and 20 μM D-PDMP (Figure 1A, black colored lines). Results from neurons exposed to D-PDMP demonstrated a similar response pattern and morphology to those treated with MBC, whereby disruption of membrane rafts resulted in neuritic retraction prior to the appearance of cell death. Given that 5 mM of MBC resulted in both neuritic retraction and cell death, and that 5 mM is frequently used experimentally (*14,17,25*), subsequent experiments were performed using this paradigm.

3.2. *Neuritic retraction occurs prior to neuronal death following exposure with MBC*

The relationship between membrane raft integrity, neuritic retraction and neuronal death was further examined using time-lapse microscopy. To do this, cortical neurons were acclimated for 30 min to the time-lapse microscope chamber and followed by incubation with 30 μg/mL PI for 30 min. PI serves as an indicator for changes of membrane permeability and neuronal death. Following acclimation and incubation with PI, cortical neurons were exposed to 5 mM MBC. Cultures were imaged using time-lapse microscopy imaging every 20 sec following MBC exposure for up to 1 h. During each time interval, both phase contrast and PI fluorescent images were obtained in Figure 2A and the supplemental Movie 1 (*http://www.biosciencetrends.com/docindex.php?year=2012&kanno=4*). Neuritic retraction occurred as early as 5 min following exposure to 5 mM of MBC and continued throughout the exposure period. The neurons only became PI positive once the majority of cell retraction occurred (Figure 2A and the supplemental Movie 1). The relationship between neuritic retraction and neuronal cell death was quantified at various time points following exposure to MBC. Neuronal viability was assessed using both a morphometric analysis of Hoechst 33258 stained nuclei and a plate-reader based Alamar blue assay. Both measurements indicated that neuritic retraction preceded neuronal death (Figure 2B). In fact, neurites retracted significantly compared to the controls as early as 5 min following exposure with MBC (70.0 ± 3.8% of control), whereas neuronal death did not occur until 20 min (88.6 ± 1.6% of control by Alamar blue assay) and 30 min (83.4 ± 2.1% of control by Hoechst morphometric analysis) (Figure 2B). Therefore, these results demonstrated that membrane raft destabilization *via* cholesterol extraction resulted in early neuritic retraction prior to the appearance of neuronal death.

3.3. *Cytoskeletal proteins are associated with membrane rafts*

Since membrane raft disruption resulted in neuritic retraction, we sought to examine the potential mechanisms by which the retraction occurred. We hypothesized that perturbation of membrane raft-associated cytoskeleton proteins may be required. To examine this possibility, the presence of neuronal cytoskeletal proteins in the membrane rafts was examined. These proteins are microtubule associated protein 2 (MAP-2), β-III-tubulin and Tau. Membrane raft fractions were isolated using sucrose gradient ultracentrifugation (see Methods section) and confirmed using immunoblotting to detect Caveolin-1 and Flotillin-1, which are makers for membrane rafts (Figure 3A). Both membrane raft and non-membrane-raft fractions were also probed by Western blotting to detect the dendritic expressed MAP-2, dendritic and axonal expressed β-III-tubulin and the axonal expressed Tau. All three microtubule associated proteins were present within the membrane raft fractions (Figure 3A). Quantitative analysis showed that the ratio of β-III-tubulin and Tau within the raft domains compared to the non-raft domains was higher than that of MAP-2. Co-localization of membrane rafts with cytoskeletal proteins was also confirmed using immunostaining of cytoskeletal proteins (red color) combined with CTxB (green color) as shown in Figure 3B.

3.4. *Stabilizing cytoskeleton does not prevent MBC-induced neurite retraction*

Experiments were performed to understand whether the observed neuritic retraction was caused by the general destabilization of microtubules in response to MBC treatment, or caused by the loss of membrane rafts which may serve as anchors for microtubule proteins. To assess these possibilities, neurons were pre-incubated with 100 nM of Paclitaxel (Taxol), a microtubule stabilizing agent, for 2 h (Figure 3C), a concentration and time point that has been shown to be protective against toxic insult (*23*). Cells pre-incubated with or without 100 nM Paclitaxel (and concentrations up to 10 μM, data not shown) demonstrated a similar rapid response with respect to neuritic retraction (Figure 3C, black colored lines) and neuronal viability (Figure 3C, red colored lines), suggesting that neuritic retraction was caused by the loss of membrane rafts serving as anchoring points for microtubule, rather than due to the destabilization of microtubules in response to MBC.

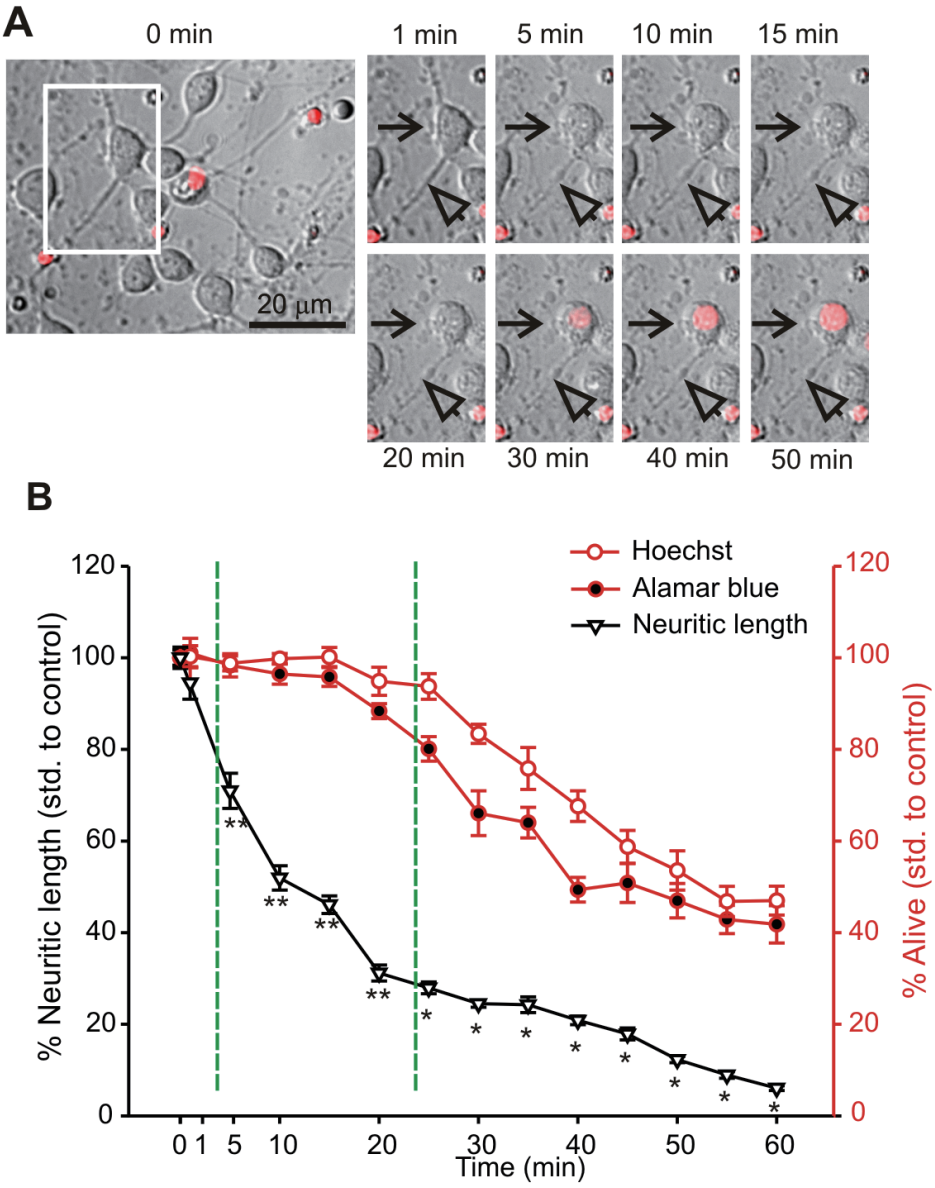

Figure 2. Time lapse imaging neuritic retraction and neuronal death following exposure to MBC. To visualize real time neuritic retraction and neuronal cell death, time-lapse microscopy was performed on 8 DIV cortical neurons treated with 30 μg/mL PI for 30 min prior to exposure with 5 mM MBC for up to 1 h. **(A)** Photomicrographs of the time-lapse imaging demonstrated neuritic retraction as early as 5 min. Cells did not become positive with PI after at least 20 min when the majority of the neurites have retracted (bar represents 20 μm). **(B)** A time course of cell viability was assessed using Hoechst cell counts and the Alamar Blue assay along with corresponding measurements of neuritic length. Cell viability was significantly reduced by 20 min (Alamar blue) and 30 min (Hoechst cell counts) following exposure to MBC. Quantitative data from two independent experiments are represented as mean changes standardized to the control untreated cells. Green lines are to highlight the difference between cell death and neuritoc length with * and ** indicating statistical significant changes from controls at $p < 0.05$ and 0.01, respectively, by one-way ANOVA, Tukey's *post hoc* test, $n = 16$.

4. Discussion

Here, we demonstrated the importance of membrane rafts in mediating neuritic retraction using two membrane raft disruption chemical agents. When membrane rafts are disrupted, perturbations of membrane raft-associated cytoskeleton proteins occur, neurites retract rapidly and neuronal death ensues. Our findings are consistent with a previous study in which inhibition of hydroxymethylglutaryl coenzyme A reductase and cholesterol synthesis in the mevalonate pathway resulted in significant neurite retraction in neuronal differentiated PC12 cells and rat cortical neurons (*26*). These findings are important in the context of understanding the pathogenesis of neuronal injury in several neurodegenerative diseases. Changes in synaptic activity caused by neuritic retraction have been postulated as being an early marker of neuronal dysfunction, commonly found early during the pathogenesis of Alzheimer's disease (*27-29*), Parkinson's disease (*30-32*) and stroke (*33*). It has also been demonstrated that neuritic retraction occurs in response to brain injury

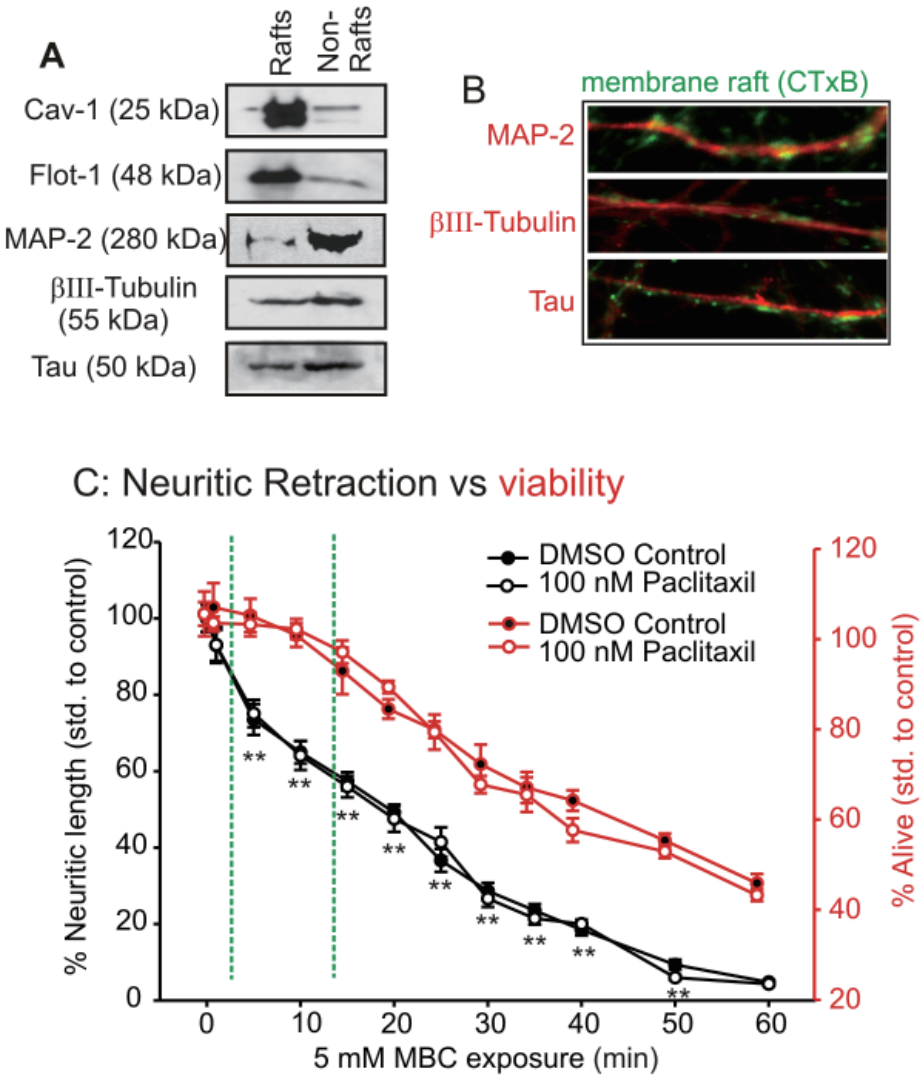

Figure 3. Detection of neuronal microtubule associated proteins within the membrane raft domains of primary cortical neurons. (A) Membrane rafts were isolated (see methods) from (DIV 8) primary cortical neurons. Membrane raft fractions were identified with Caveolin-1 and Flotillin-1 by immunoblotting. Both membrane raft and non-raft fractions were also probed by immunoblotting with the dendritic marker MAP-2, dendritic and axonal marker β-III-tubulin and the axonal marker Tau. All three microtubule associated proteins were present both within the membrane raft and non-raft fractions. The ratio of β-III-tubulin and Tau within the raft fractions compared to the non-raft fractions was higher than MAP-2. **(B)** Primary cortical neurons were also fixed and labelled with CTxB (green color), followed by immunolabelling with antibodies to MAP-2, β-III-tubulin or Tau (red color). **(C)** Effects of MBC on neuritic retraction and viability following treatment with Paclitaxel. Cortical neurons at 8 DIV were pre-incubated with either DMSO or 100 nM Paclitaxel for 2 h. Neurons were then exposed to 5 mM MBC for up to 1 h. Neuritic length was measured at various time points along with corresponding measurements of cell viability assessed using the Alamar Blue. There were no statistical significant differences in neuritic retraction or neuronal viability between neurons pre-incubated with DMSO alone or Paclitaxel (one-way ANOVA with Tukey's *post hoc* test, $n = 10$). Green lines are to highlight the difference between cell death and neurite length with ** indicating statistical significant changes at $p < 0.01$ between neuritic length and percentage of live cells (one-way ANOVA, Tukey's *post hoc* test, $n = 16$).

(*28,34-36*). Together, our data clearly demonstrates that neuritic retraction is mediated by membrane rafts and further suggests that such a mechanism may potentially be common in neuritic response to injury.

Our data clearly established the fact that disruption of membrane rafts results in neuritic retraction prior to neuronal death. Using morphometric analysis and time-lapse imaging, and two membrane raft-disrupting agents, we convincingly showed that neuritic retraction occurred prior to the appearance of PI positivity of the nuclei, confirming neuronal death. Interestingly, some studies have suggested that the inhibition of membrane raft function is neuroprotective (*37,38*). These particular studies suggest that by not allowing clustering of potential harmful receptors such as the N-methyl-D-aspartic acid receptor into rafts, neurons could be protected against excitotoxicity. Our results indicate that MBC caused significant neuritic retraction even at low doses (5 μM). While our results do not refute previous claims that preventing potentially harmful signaling through membrane rafts may provide neuroprotection, they also suggest that a complete disruption of membrane rafts *via*

cholesterol depletion leads to neurodegeneration. This notion that a quantum of membrane rafts are required for neuroprotection following injury is supported by recent studies demonstrating that cytidine 5-diphosphocholine redistributed excitatory amino acid transporters to membrane raft microdomains and improved glutamate uptake in a rat middle cerebral artery occlusion model (*39,40*). More recently, it has been demonstrated that excitatory amino acid transporter 2 localization was significantly reduced within membrane raft domains of Alzheimer's disease patients (*41*). Overall, these studies, together with our results, suggest that membrane rafts are required for neuritic integrity and neuronal viability.

Since MBC exposure resulted in rapid neuritic retraction, examining microtubule associated protein response to MBC was done. Western Blotting results demonstrated that the microtubule proteins, MAP-2, β-III-tubulin and Tau are all present within the membrane raft domains of primary cortical neurons. Using Immunocytochemistry, CTxB-labelled membrane rafts appeared to localize with the dendritic microtubule protein, MAP-2, in roughly equal amounts within the neurites and cell bodies, higher amounts within the neurites with β-III-tubulin, and almost exclusively within the neurites with Tau. The most common preparation for membrane-raft associated protein is the Triton X-100/sucrose density gradient centrifugation method. Cognisant that this technique is not without faults (*42-44*), we were confident that MAP-2, β-III-tubulin and Tau were co-localized within these domains since they were confirmed both using the biochemical Triton X-100/ sucrose density gradient method and by labeling with CTxB.

Disruption of membrane rafts with MBC leads to rapid de-colocalization of membrane rafts, in order of increased rate, with MAP-2, β-III-tubulin and Tau, all of which suggests that axonal microtubules may be more sensitive to membrane raft disruption. The crucial point in this finding lies within the timing. The shift in colocalization between membrane rafts and microtubule associated proteins occurred prior to neuritic retraction and cell death. Our results are consistent with other studies which have demonstrated that cholesterol containing membrane rafts interact with components of the cytoskeleton (*8-10*). To our knowledge, however, this is the first study that has described the presence of both dendritic and axonal microtubule proteins within membrane rafts. Further research needs to be conducted to examine the structural supportive role of membrane rafts in neurites. A clear understanding of the mechanisms involved in this structural support may serve as a novel neuroprotective strategy for neurons exposed to injury.

Acknowledgements

We thank the IBS Animal Facility for the timely supply of experimental animals. This research is supported by the Heart and Stroke Foundation of Ontario grant T5760 and T6706 to S.T.H..

References

1. Pike LJ. Rafts defined: A report on the Keystone Symposium on Lipid Rafts and Cell Function. J Lipid Res. 2006; 47:1597-1598.
2. Pike LJ. The challenge of lipid rafts. J Lipid Res. 2009; 50 (Suppl S):323-328.
3. Hering H, Lin CC, Sheng M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J Neurosci. 2003; 23:3262-3271.
4. Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ. Lipid rafts mediate chemotropic guidance of nerve growth cones. Neuron. 2004; 42:51-62.
5. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000; 1:31-39.
6. Korade Z, Kenworthy AK. Lipid rafts, cholesterol, and the brain. Neuropharmacology. 2008; 55:1265-1273.
7. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997; 387:569-572.
8. Itoh K, Sakakibara M, Yamasaki S, Takeuchi A, Arase H, Miyazaki M, Nakajima N, Okada M, Saito T. Cutting edge: Negative regulation of immune synapse formation by anchoring lipid raft to cytoskeleton through Cbp-EBP50-ERM assembly. J Immunol. 2002; 168:541-544.
9. Foger N, Marhaba R, Zoller M. Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells. J Cell Sci. 2001; 114:1169-1178.
10. Dremina ES, Sharov VS, Schoneich C. Protein tyrosine nitration in rat brain is associated with raft proteins, flotillin-1 and alpha-tubulin: Effect of biological aging. J Neurochem. 2005; 93:1262-1271.
11. Kawabe Ji, Okumura S, Nathanson MA, Hasebe N, Ishikawa Y. Caveolin regulates microtubule polymerization in the vascular smooth muscle cells. Biochemical and Biophysical Research Communications. 2006; 342:164-169.
12. Levitan I, Gooch KJ. Lipid rafts in membrane-cytoskeleton interactions and control of cellular biomechanics: Actions of oxLDL. Antioxid Redox Signal. 2007; 9:1519-1534.
13. Abulrob A, Tauskela JS, Mealing G, Brunette E, Faid K, Stanimirovic D. Protection by cholesterol-extracting cyclodextrins: A role for N-methyl-D-aspartate receptor redistribution. J Neurochem. 2005; 92:1477-1486.
14. Bar-On P, Rockenstein E, Adame A, Ho G, Hashimoto M, Masliah E. Effects of the cholesterol-lowering compound methyl-beta-cyclodextrin in models of alpha-synucleinopathy. J Neurochem. 2006; 98:1032-1045.
15. Huang P, Xu W, Yoon SI, Chen C, Chong PL, Liu-Chen LY. Cholesterol reduction by methyl-beta-cyclodextrin attenuates the delta opioid receptor-mediated signaling in neuronal cells but enhances it in non-neuronal cells. Biochem Pharmacol. 2007; 73:534-549.
16. Gibson NJ, Tolbert LP, Oland LA. Roles of specific membrane lipid domains in EGF receptor activation and cell adhesion molecule stabilization in a developing olfactory system. PLoS One. 2009; 4:e7222.
17. Sjogren B, Csoregh L, Svenningsson P. Cholesterol reduction attenuates 5-HT1A receptor-mediated

signaling in human primary neuronal cultures. Naunyn Schmiedebergs Arch Pharmacol. 2008; 378:441-446.
18. Sillence DJ, Puri V, Marks DL, Butters TD, Dwek RA, Pagano RE, Platt FM. Glucosylceramide modulates membrane traffic along the endocytic pathway. J Lipid Res. 2002; 43:1837-1845.
19. Szoke E, Borzsei R, Toth DM, Lengl O, Helyes Z, Sandor Z, Szolcsanyi J. Effect of lipid raft disruption on TRPV1 receptor activation of trigeminal sensory neurons and transfected cell line. Eur J Pharmacol. 2010; 628:67-74.
20. Sjogren B, Svenningsson P. Depletion of the lipid raft constituents, sphingomyelin and ganglioside, decreases serotonin binding at human 5-HT7(a) receptors in HeLa cells. Acta Physiologica. 2007; 190:47-53.
21. Giovanni A, Keramaris E, Morris EJ, Hou ST, O'Hare M, Dyson N, Robertson GS, Slack RS, Park DS. E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. J Biol Chem. 2000; 275:11553-11560.
22. Hou ST, Jiang SX, Desbois A, Huang D, Kelly J, Tessier L, Karchewski L, Kappler J. Calpain-cleaved collapsin response mediator protein-3 induces neuronal death after glutamate toxicity and cerebral ischemia. J Neurosci. 2006; 26:2241-2249.
23. Michaelis ML, Ranciat N, Chen Y, Bechtel M, Ragan R, Hepperle M, Liu Y, Georg G. Protection against beta-amyloid toxicity in primary neurons by paclitaxel (Taxol). J Neurochem. 1998; 70:1623-1627.
24. Fatokun AA, Stone TW, Smith RA. Adenosine receptor ligands protect against a combination of apoptotic and necrotic cell death in cerebellar granule neurons. Exp Brain Res. 2008; 186:151-160.
25. Bruses JL, Chauvet N, Rutishauser U. Membrane lipid rafts are necessary for the maintenance of the (alpha)7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons. J Neurosci. 2001; 21:504-512.
26. Schulz JG, Bosel J, Stoeckel M, Megow D, Dirnagl U, Endres M. HMG-CoA reductase inhibition causes neurite loss by interfering with geranylgeranylpyrophosphate synthesis. J Neurochem. 2004; 89:24-32.
27. Leuba G, Walzer C, Vernay A, Carnal B, Kraftsik R, Piotton F, Marin P, Bouras C, Savioz A. Postsynaptic density protein PSD-95 expression in Alzheimer's disease and okadaic acid induced neuritic retraction. Neurobiol Dis. 2008; 30:408-419.
28. Levin EC, Acharya NK, Sedeyn JC, Venkataraman V, D'Andrea MR, Wang HY, Nagele RG. Neuronal expression of vimentin in the Alzheimer's disease brain may be part of a generalized dendritic damage-response mechanism. Brain Res. 2009; 1298:194-207.
29. Selkoe DJ. Alzheimer's disease is a synaptic failure. Science. 2002; 298:789-791.
30. Plowey ED, Cherra SJ, III, Liu YJ, Chu CT. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. J Neurochem. 2008; 105:1048-1056.
31. MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A. The familial Parkinsonism gene *LRRK2* regulates neurite process morphology. Neuron. 2006; 52:587-593.
32. Farrer MJ. Genetics of Parkinson disease: Paradigm shifts and future prospects. Nat Rev Genet. 2006; 7:306-318.
33. Hou ST, Keklikian A, Slinn J, O'Hare M, Jiang SX, Aylsworth A. Sustained up-regulation of semaphorin 3A, Neuropilin1, and doublecortin expression in ischemic mouse brain during long-term recovery. Biochem Biophys Res Commun. 2008; 367:109-115.
34. Flood DG, Coleman PD. Dendritic regression dissociated from neuronal death but associated with partial deafferentation in aging rat supraoptic nucleus. Neurobiol Aging. 1993; 14:575-587.
35. Terry RD, Katzman R. Life span and synapses: Will there be a primary senile dementia? Neurobiol Aging. 2001; 22:347-348.
36. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. Ann Neurol. 1991; 30:572-580.
37. van der Most PJ, Dolga AM, Nijholt IM, Luiten PG, Eisel UL. Statins: Mechanisms of neuroprotection. Prog Neurobiol. 2009; 88:64-75.
38. Ponce J, de la Ossa NP, Hurtado O, Millan M, Arenillas JF, Davalos A, Gasull T. Simvastatin reduces the association of NMDA receptors to lipid rafts: A cholesterol-mediated effect in neuroprotection. Stroke. 2008; 39:1269-1275.
39. Hurtado O, Moro MA, Cardenas A, Sanchez V, Fernandez-Tome P, Leza JC, Lorenzo P, Secades JJ, Lozano R, Davalos A, Castillo J, Lizasoain I. Neuroprotection afforded by prior citicoline administration in experimental brain ischemia: Effects on glutamate transport. Neurobiol Dis. 2005; 18:336-345.
40. Hurtado O, Pradillo JM, Fernandez-Lopez D, Morales JR, Sobrino T, Castillo J, Alborch E, Moro MA, Lizasoain I. Delayed post-ischemic administration of CDP-choline increases EAAT2 association to lipid rafts and affords neuroprotection in experimental stroke. Neurobiol Dis. 2008; 29:123-131.
41. Tian G, Kong Q, Lai L, Ray-Chaudhury A, Liang Glenn LC. Increased expression of cholesterol 24S-hydroxylase results in disruption of glial glutamate transporter EAAT2 association with lipid rafts: A potential role in Alzheimer's disease. J Neurochem. 2010; 113:978-989.
42. Munro S. Lipid rafts: Elusive or illusive? Cell. 2003; 115:377-388.
43. Hancock JF. Lipid rafts: Contentious only from simplistic standpoints. Nat Rev Mol Cell Biol. 2006; 7:456-462.
44. Babiychuk EB, Draeger A. Biochemical characterization of detergent-resistant membranes: A systematic approach. Biochem J. 2006; 397:407-416.

(*Received July 11, 2012; Revised August 15, 2012; Accepted August 16, 2012*)

Legend for Supplementary Movie 1

Neuritic retraction and neuronal death in response to MBC by time lapse imaging. Primary cortical neurons were acclimated for 30 minutes to the time lapse chamber and were maintained at 37°C with 5 % CO_2. Cells were then incubated with 30 μg/mL propidium iodide (PI) for 30 min. Following acclimation and incubation with PI, cortical neurons were exposed to 5 mM MBC. Cultures were imaged using time lapse microscopy every 20 seconds following MBC exposure for up to 1 h. During each time interval both phase and Cy-3 images were obtained. The appearance of the red color indicates PI positivity and cell death.