Cholesterol rich, highly phase separated, naked mole-rat brain lipids are exquisitely sensitive to amyloid induced membrane damage

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Abstract:
Naked mole-rats do not develop neurodegenerative diseases associated with ageing and their brains are devoid of amyloid plaques. However, even the young naked mole-rat brain contains high concentrations of amyloid beta peptide. Thus, the question arises, how do naked mole-rat brain cell membranes survive in this amyloid rich environment? In this work we examine the composition, phase behaviour, and amyloid beta interactions of naked mole-rat brain lipids. Relative to mouse, naked mole-rat brain lipids are rich in cholesterol and contain less sphingomyelin, the sphingomyelin present being of a characteristic short chain length. We find that naked mole-rat brain lipid membranes exhibit an extremely high degree of phase separation, consistent with the membrane pacemaker hypothesis of ageing, with the liquid ordered phase occupying up to 80 % of the supported lipid bilayer. Exposure of mouse brain lipids to human amyloid beta at physiologically relevant concentrations leads to small, well-defined "footprints", whereby the amyloid beta has sunk into the membrane. Under the same exposure regime, the naked mole-rat brain lipid membranes are destroyed, leaving only membrane fragments in place of the intact membrane. These results suggest that naked mole-rats have likely developed additional neuroprotective mechanisms to limit cellular damage because by considering lipids alone brain tissue should not be able to survive in such a toxic environment.

Introduction

The naked mole-rat is a long-lived species having a maximum life span in excess of 30 years[1,2] (comparable sized rodents having a maximum lifespan of 3-4 years). Possessing many neotenic features[3,4] and defying many of the signs of ageing[5], the naked mole-rat does not appear to develop neurodegenerative disease[6,7]. Considering the interest in amyloid beta as an age-related pathogenic moiety[8], it was perhaps surprising that the naked mole-rat brain was observed to have high levels of amyloid beta (exceeding those found in 3×Tg-AD mice) even in the brains of young animals, thus suggesting that there are no age-related changes in amyloid beta levels as reported by Edrey et al[6]. Somehow naked mole-rats are able to live for years with this potentially neurotoxic peptide. The exact role of amyloid beta in Alzheimer's disease (AD) remains controversial[9] with numerous drugs targeting amyloid pathways failing in clinical trials. However, whether a bystander or an active participant in disease progression, amyloid plaques are a hallmark of the diseased brain. Edrey et al[6] also reported that no such amyloid plaques were found in naked mole-rat brains. Thus, the question remains, why is it that amyloid plaques do not form in naked mole-rat brains? The first port of call might be to look at the differences between human and naked mole-rat amyloid beta. However, there is only a single amino acid difference between human and naked mole-rat amyloid beta, which Edrey et al. found caused a difference in aggregation kinetics, but did not alter the degree of toxicity to mouse hippocampal neurones[6]. Another possible explanation may be found in the cell membrane given the role of the membrane in amyloid assembly and as a target of amyloid pore formation[10-12,13]. For example, there is a strong link between cholesterol and Alzheimer's disease, which could be related to amyloid-membrane interactions[14-16]. Naked mole-rat lipids from brains and other tissue, have been analysed in relation to their role in the membrane pacemaker theory of ageing, which states that long-lived species will have membrane compositions that resist peroxidation, a proposed mechanism in resistance to ageing. In naked mole-rat brains and within other tissues, it was observed that there was significantly less docosahexanoic acid (DSA), a fatty acid which is particularly susceptible to peroxidation, than that found in mice . In addition, it was reported that naked mole-rat membranes contain more vinyl ether linked phospholipids (plasmalogens) which are resistant to peroxidation.[17,18] Plasmalogens are highly enriched in lipid rafts/microdomains and contribute to their stability.[19] Thus, a high level of this ether-lipid may be an indicator of a high raft content, or increased raft stability.[20] Atomic force microscopy (AFM) presents many advantages for examining lipid bilayer based systems, in particular its ability to quantify two dimensional ordering and to follow interactions of the membrane with peptides in real time[21,22]. In this work, we use a combination of lipidomics and AFM to characterise naked mole-rat brain lipids in terms of composition and two dimensional ordering. Comparisons are made with brain lipids extracted from the short-lived, similar sized mouse. We then use AFM to follow the interactions of synthetic human amyloid beta with brain derived lipid membranes from both mouse and naked mole-rat. The membrane pacemaker theory of ageing suggests that cell membranes of long-lived species will possess more peroxidation resistant species which may also be raft forming[17]. We thus hypothesise that naked mole-rat brain derived lipid bilayers will show a high degree of phase separation (two-dimensional ordering) and that there may be a unique lipid signature that "protects" the membranes against amyloid beta induced damage.

Results and Discussion

Mass spectrometry of brain total lipid extract shows that there are key differences between naked mole-rat and mouse brains, which may have implications for amyloid beta assembly and membrane interactions. Firstly, naked mole-rat brains have significantly more free cholesterol than mouse (Figure 1A). Also of note is the relatively low level of sphingomyelin in naked mole-rat brains relative to mouse (Figure 1B). Pettegrew et al. found that the levels of brain sphingomyelin positively correlate with amyloid beta plaques, however other studies show the opposite correlation between sphingomyelin and AD[23]. In contrast to the observations for free cholesterol and sphingomyelin, the concentrations of the total phospholipids and ceramides were not different between naked mole-rat and mouse brains (Figure 1C&D). The relative abundances of these different lipid species were evaluated and presented as heatmaps (Figure 1E&F) and as tables (supplementary table 1-6). We found that these lipidomic profiles were different between mouse and naked mole-rat brains as illustrated by a clustering of mouse and naked mole-rat depending on the number of carbons of the phospholipids, or of the sphingomyelins and ceramides (Figure 1E&F, top dendrograms). Concerning the phospholipids, four major groups of lipids were identified:

Cluster 1 contained lipids which were more abundant in naked mole-rat than mouse brains, Clusters 2 and 3 contained lipids which were not differentially expressed between mouse and naked mole-rat brains, and Cluster 4 contained lipids that were more abundant in mouse brains (Figure 1E). However, these four clusters did not reveal a distinct pattern of species. The ceramide and sphingolipids clustering highlighted 4 clusters: Cluster 1 was composed of lipids more abundant in mouse brains, Cluster 3 by lipids more abundant in naked mole-rat brains, and Clusters 2 and 4 being no different between the two species. Interestingly, the sphingomyelins and ceramides preferentially presented in naked mole-rat brains (Cluster 3) were the shortest lipids quantified. In conclusion, our lipid analysis revealed differences in the lipid composition of mouse and naked mole-rat brains. Overall, naked mole-rat brain lipids contained a higher concentration of cholesterol and lower concentration of sphingomyelins, which were of shorter length than those present in mouse brains, but contained a higher percentage of saturated sphingomyelin (Supp. Figure 1). We completed the comparison of lipid composition of naked mole-rat and mouse brains by quantifying the concentration in fatty acids (FA). No significant differences were observed between the concentrations of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) in mouse and naked mole-rat brains (Figure 2A, B & C). However, the n-6/n-3 ratio of PUFAs was increased in naked mole-rat brains compared to mouse brains. The relative abundances of these FA were evaluated and presented as a heatmap (Figure 2E) and as table (supplementary table 7). We found that the FA profile was different between mouse and naked mole-rat brains, illustrated by a clustering of mouse and naked mole-rat depending on the number of carbons within the FA (Figure 2E). Three major groups of FA were identified: Cluster 1 contained lipids which were more abundant in mouse brains, Cluster 2 contained FA which were not differentially expressed between mouse and naked mole-rat brains, and Cluster 3 contained lipids that were more abundant in naked mole-rat brains (Figure 2F). As observed for the ceramides and sphingomyelin, the SFA and MUFA abundant in naked mole-rat brains (Cluster 3) were of shorter length than those present in mouse brains. Supplementary Tables 1 -7 provide a detailed comparative overview of the percentage of different lipid species identified in mouse and naked mole-rat brain lipids.

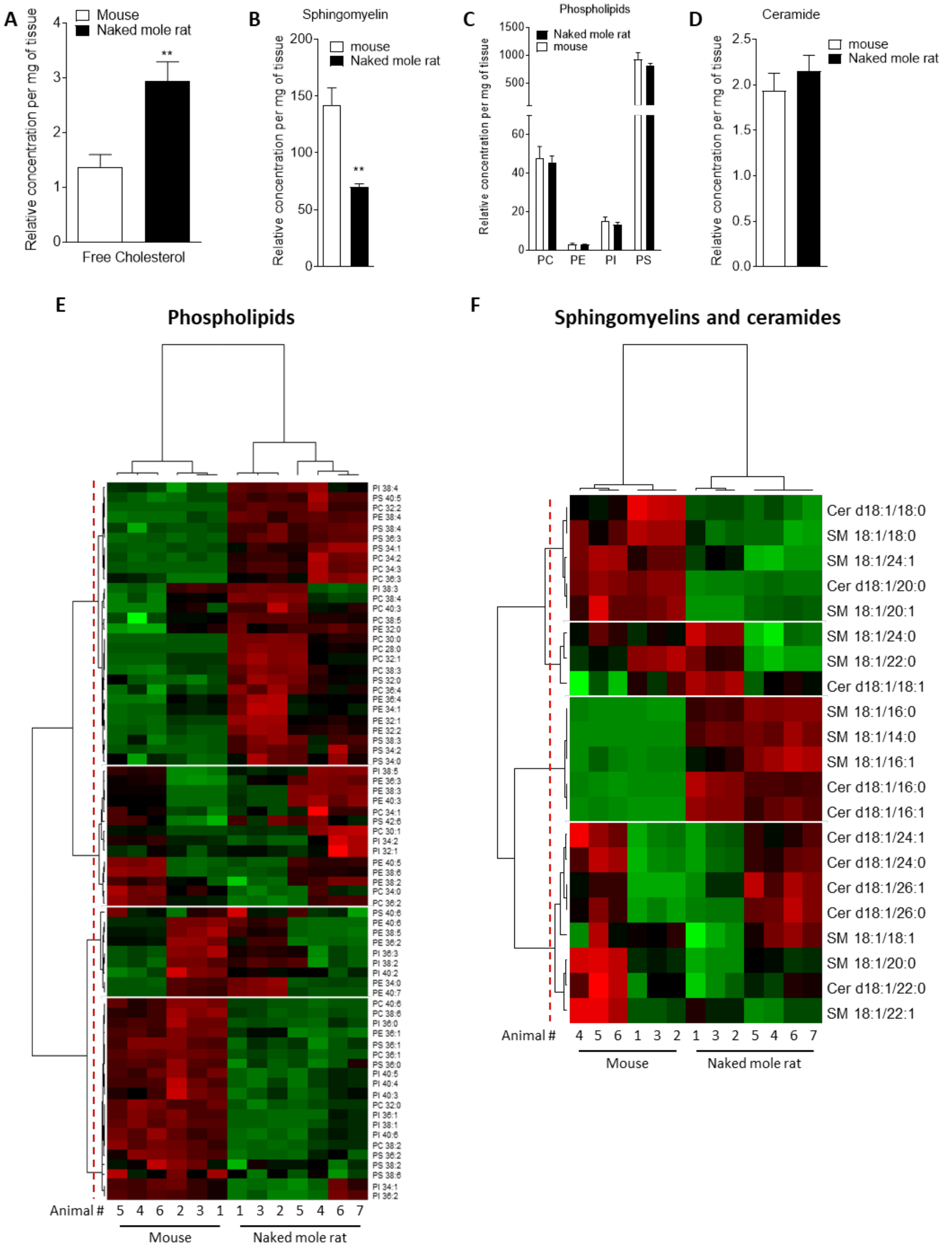

Figure 1 – Mass Spectrometry of brain derived total lipid extract. Lipids were extracted from the brains of 7 naked mole-rats and 6 mice (2 independent experiments). Quantification of (A) free cholesterol, (B) sphingomyelin, (C) phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), and (D) ceramides in lipid extract from mouse (white bar) or naked mole-rat (black bar) brains. Data are expressed as mean ± SEM. Statistical analysis was performed using the Mann-Whitney test. **p<0.01, significantly different from mouse group. (E) Heat map of the different phospholipids quantified by LC-MS/MS. Data are shown in a

matrix format: each row represents a single phospholipid, and each column represents the lipid extract of the brain of one animal (6 mice and 7 naked mole-rat). Each colour patch represents the normalized quantity of phospholipid (row) in a single animal brain (column), with a continuum of quantity from bright green (lowest) to bright red (highest). The pattern and length of the branches in the left dendrogram reflect the relatedness of the phospholipids. The dashed red line is the dendrogram distance used to cluster the phospholipids. The pattern and length of the branches in the top dendrogram reflect the relatedness of the different animals. (F) Heat map of the different sphingomyelins and ceramides quantified by LC-MS/MS. Data are shown in a matrix format: each row represents a single sphingomyelin or ceramide, and each column represents the lipid extract of the brain of one animal (6 mice and 7 naked mole-rat). Each colour patch represents the normalized quantity of sphingomyelin or ceramide (row) in a single animal brain (column), with a continuum of quantity from bright green (lowest) to bright red (highest). The pattern and length of the branches in the left dendrogram reflect the relatedness of the sphingomyelins and ceramides. The dashed red line is the dendrogram distance used to cluster the phospholipids. The pattern and length of the branches in the top dendrogram reflect the relatedness of the different animals.

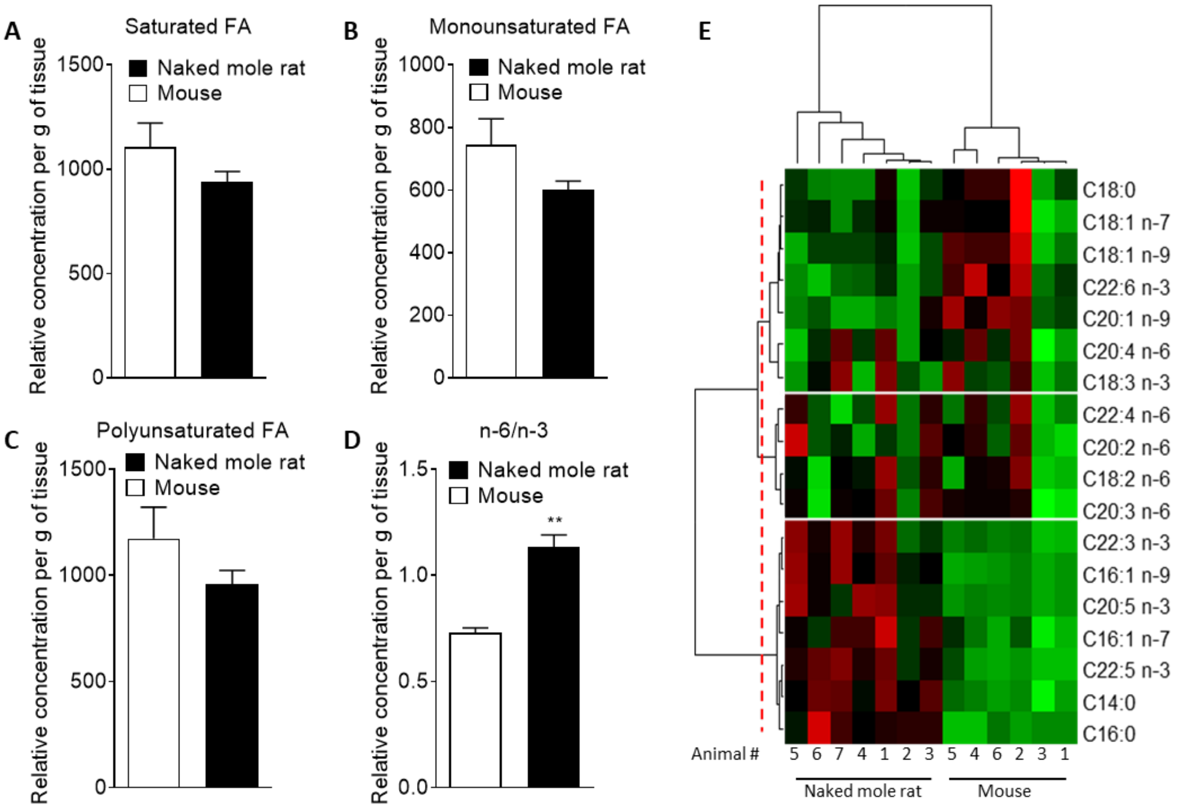

Figure 2: Mass Spectrometry of brain derived total fatty acids. Lipids were taken from the brains of 7 naked mole-rats and 6 mice (2 independent experiments). Quantification of (A) saturated fatty acids, (B) monounsaturated fatty acids, (C) polyunsaturated fatty acids (PUFA), and (D) n-6 PUFA/n-3 PUFA ratio extract from mouse (white bar) or naked mole-rat (black bar) brains. Data are expressed as mean ± SEM. Statistical analysis was performed using Mann-Whitney test. **$p<0.01$, significantly different from mouse group. (E) Heat map of the different fatty acids (FA) quantified by LC-MS/MS. Data are shown in a matrix format: each row represents a single FA, and each column represents the lipid extract of the brain of one animal (6 mice and 7 naked mole-rat). Each colour patch represents the normalized quantity of FA (row) in a single animal brain (column), with a continuum of quantity from bright green (lowest) to bright red (highest). The pattern and length of the branches in the left dendrogram reflect the relatedness of the FA. The dashed red line is the dendrogram distance used to cluster the FA. The pattern and length of the branches in the top dendrogram reflect the relatedness of the different animals.

Prior to AFM examination of interactions between human amyloid beta and the brain tissue derived lipids, we used a simple model system comprising of just two lipids, DOPC and DPPC. This system exhibits phase separation and provides a well-defined comparison for the more complex mixture of lipids present in brain derived lipids. When the binary system was mixed in a molar ratio of 3:1 of DOPC-to-DPPC, we observed the classic phase separated DPPC gel domains surrounded by the liquid disordered DOPC (Figure 3A). The origin of the "islands" of DPPC is due to the packing arrangement of DPPC chains, resulting in a height difference of around 1.5 nm to the surrounding DOPC (Figure 3B). Such spontaneous phase separation behaviour in model systems has been used as a rationale for the existence of lipid rafts in cells[24]. After 2 hours exposure to 8 μM of human amyloid beta the bilayer remained completely intact, but with clearly visible features in between the DPPC gel phase domains, that are absorbed on top of the DOPC liquid disordered phase (highlighted by blue circle in 3C). These features are the amyloid beta peptide and it is to be noted that no peptide adsorbs on the gel phase domains. Upon closer inspection, the amyloid beta is observed to avoid the gel phase domains, such that the central domain is surrounded, but not covered, by the peptide (Figure 3D).

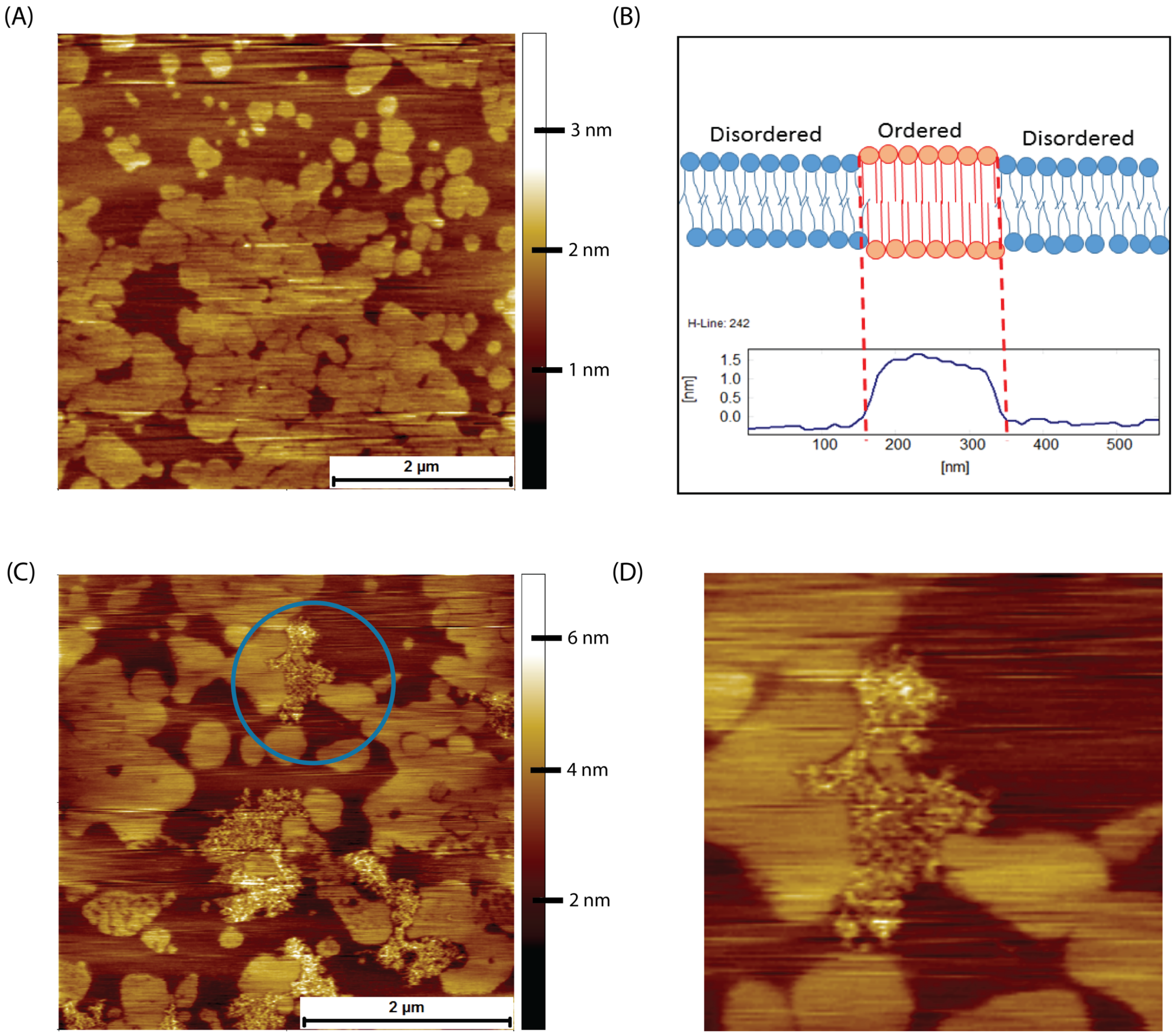

Figure 3 – AFM contact mode images (in PBS) of DOPC/DPPC model supported lipid bilayers and

their interactions with human amyloid beta peptide. A) Supported bilayer made from DOPC to DPPC in a molar ratio of 3:1. Typical phase separation is observed with the DPPC gel phase islands surrounded by the liquid disordered DOPC phase. B) Proposed origin of height difference of the gel phase relative to liquid disordered phase. C) DOPC/DPPC bilayer after exposure for 2 hours to 8 μM of human amyloid beta. The amyloid beta has adsorbed exclusively onto the DOPC liquid disordered phase, the DPPC gel phase remaining untouched. D) A zoom in of the area enclosed in the circle in (C)., the amyloid beta being seen to avoid the DPPC gel domains, even the small central gel domain remaining untouched.

Lipid bilayers formed from naked mole-rat brain derived total lipid extract were formed on a cleaved mica surface and were found to exhibit an extremely high degree of phase separation, with the characteristic "islands" Figure 4A). These islands could occupy of greater than 80% of the membrane. At higher magnification, it was sometimes possible to distinguish a two-tier raft (Figure 4B and 4C), but this was not seen in all preparations. However, the high degree of phase separation was observed in lipids extracted from all naked mole-rat brains studied. For comparison mouse brain lipids exhibited little to no phase separation, such that a lipid membrane formed with little to no deviation in height and structure (Figure 4D).

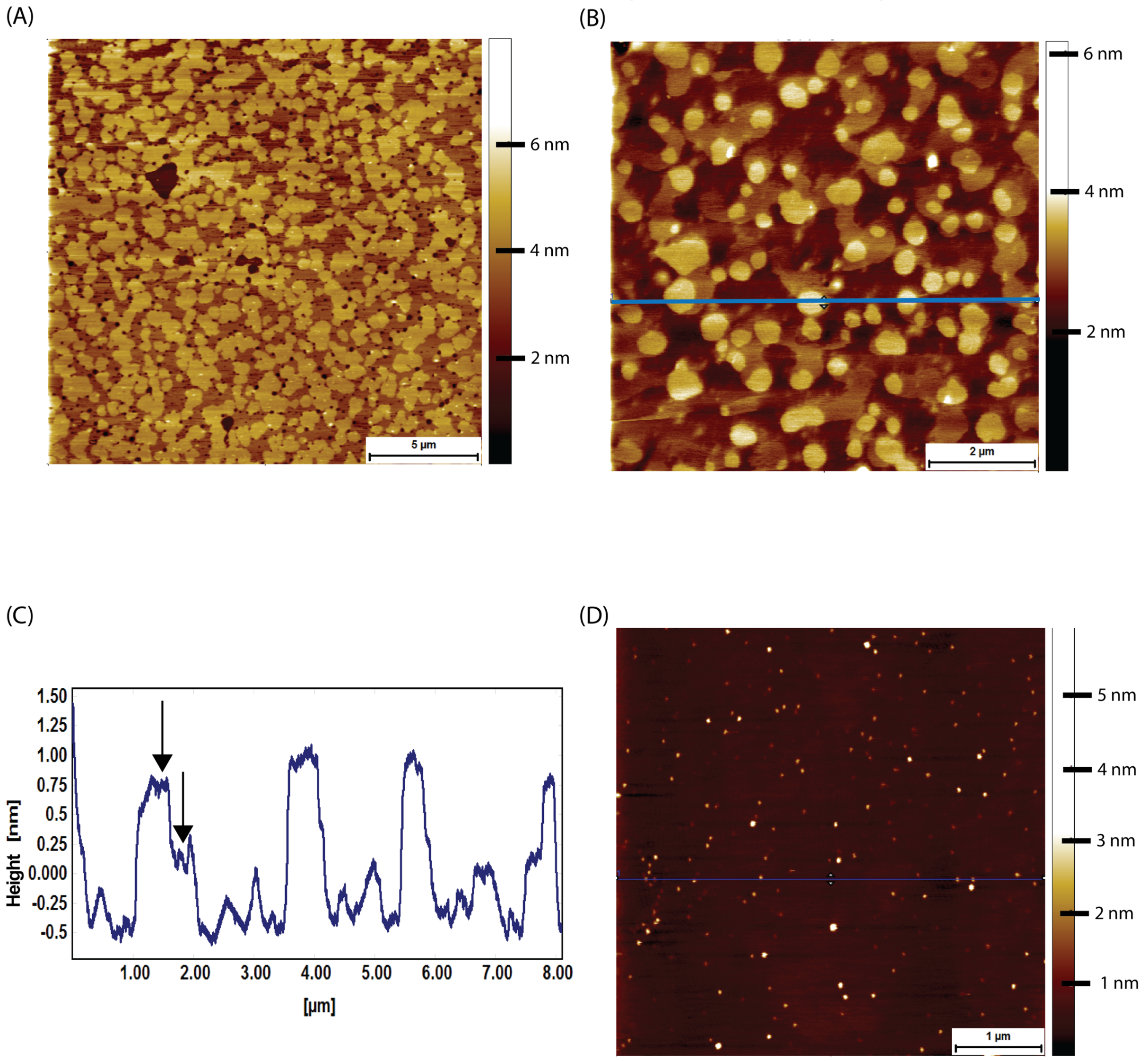

Figure 4 – AFM tapping mode images (in PBS) of brain tissue derived supported lipid bilayers. A)

Supported bilayer made from naked mole-rat brain derived lipids. More than 80 % of the membrane is made up of phase separated "islands" ~1.5 nm in height relative to the surrounding liquid disordered phase. B) Supported bilayer made of naked mole-rat brain lipids showing a two-tier raft structure, the horizontal blue line corresponding to the height profile in C, where the two-tier raft structure is evident from the peaks having a shoulder, the first at ~0.75 nm, the second at ~1.25 nm, indicated by arrows in the figure. D) Supported lipid bilayer made from mouse brain derived lipids. In contrast to the naked mole-rat bilayers there is little evidence of phase separation.

It has been hypothesised that cell membranes in naked mole-rats (along with mitochondrial and other membrane structures) might show a high proportion of lipids capable of phase separation as these raft forming species were more likely to resist lipid peroxidation and thus resist ageing[18]. The results observed here are consistent with the lipid pacemaker theory[25], with phase separated domains accounting for up to 80% of the membrane (Figure 4A). Rafts are particularly rich in sphingolipids, cholesterol, and saturated phospholipids and our lipidomic analyses revealed an enrichment in cholesterol (Figure 1A) and in sphingomyelin with a SFA (Supplementary Figure 1) associated with the sphingosine. However, the total SFA concentration was not different between mouse and naked mole-rat brain lipid extracts (Figure 2A).

By indenting the conical AFM tip into the lipid bilayer it is possible to measure the breakthrough force[26], the force required to push through the soft thin film (Figure 5A and 5B), thereby providing an indication of the membrane fluidity. It also allows the measurement of membrane thickness, which is a necessary confirmation that a thin soft bilayer is being imaged. Although no difference was observed in the thickness of lipid bilayer membranes formed from mouse or naked mole-rat brain derived lipids (Figure 5C), the breakthrough force measurements for naked mole-rat membranes were significantly higher than mouse (Figure 5D), meaning that naked mole-rat lipid bilayer membranes are "stiffer". The enhanced stiffness of naked mole-rat lipid bilayer membranes is most likely due to the increased cholesterol, which is known to increase membrane rigidity.[27]

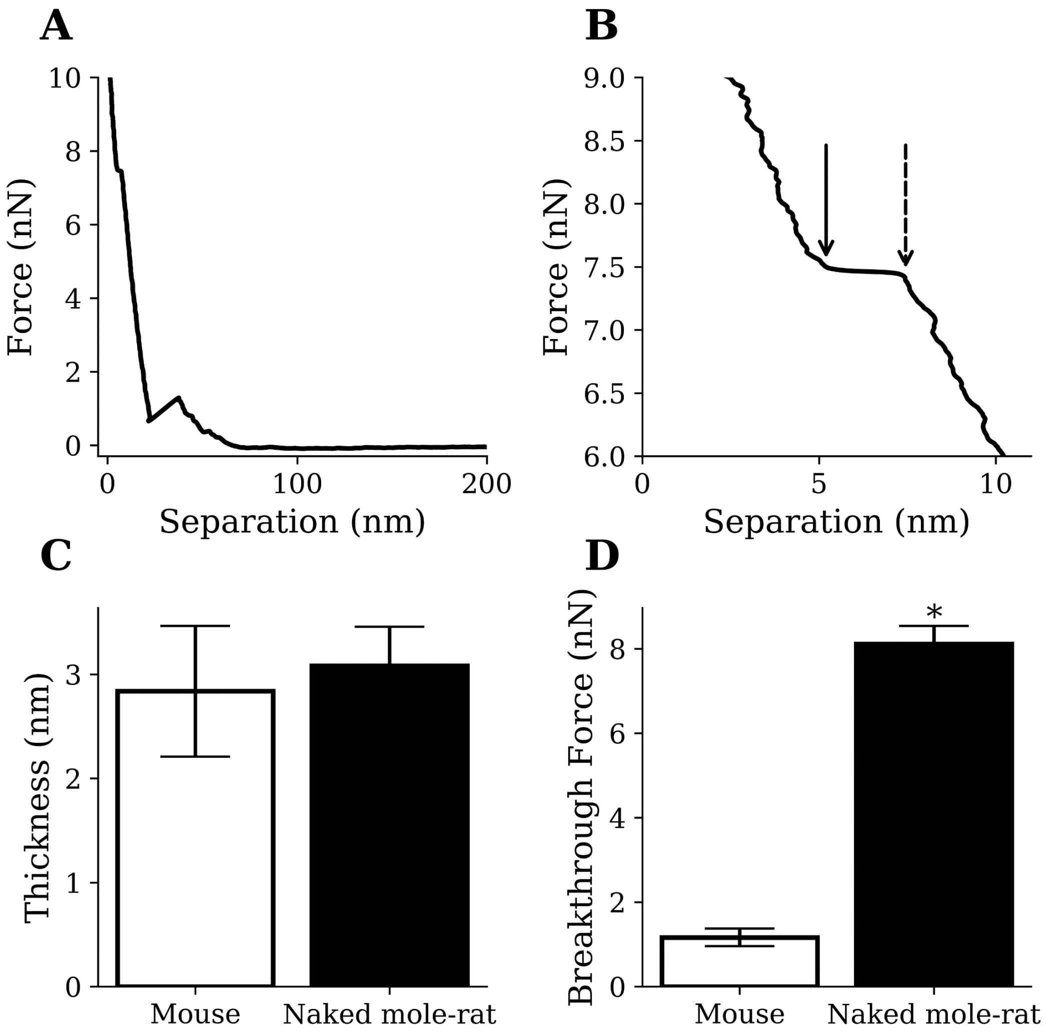

Figure 5 - AFM results from mouse sample (n=1220) and naked mole-rat sample (n=2859) lipid bilayer indentations. Example indentation on a naked mole-rat bilayer (A), where multiple breakthrough events were detected only the event closest to the mica (B) was recorded. The points indicated by the dashed and solid arrow in panel B show the start and end of the breakthrough event respectively. Thickness was recorded as the difference in separation between the points and breakthrough force was recorded at the start of the breakthrough indicated by the dashed arrow. Thickness values (C) of 3.1 nm ± 0.4 nm and 2.8 nm ± 0.6 nm were recorded for the naked mole-rat and mouse lipid bilayers respectively. Breakthrough force values (D) of 8.1 nN ± 0.4 nN and 1.2 nN ± 0.2 nN were recorded for the naked mole-rat and mouse lipid bilayers respectively. Data are expressed as mean ± SEM. Statistical analysis was performed using the unpaired t test. *p<0.05, significantly different from mouse group.

Prior to exposure to amyloid beta, the mouse brain derived lipid bilayer had a characteristic morphology exhibiting almost no phase separation with only the occasional, small domain being visible (Figure 6A). When exposed to 8 μM human amyloid beta for two hours, pores appeared in the mouse derived lipid bilayer (Figure 6B). The pores were not circular, and there was little sign of the amyloid beta that had caused them. A likely explanation is that the amyloid beta peptides have sunk into the membrane because the height profile through the pores shows that they are less deep than the thickness of the bilayer (Figure 6C and D). It is important to note that the integrity of the membrane, as a whole, is maintained.

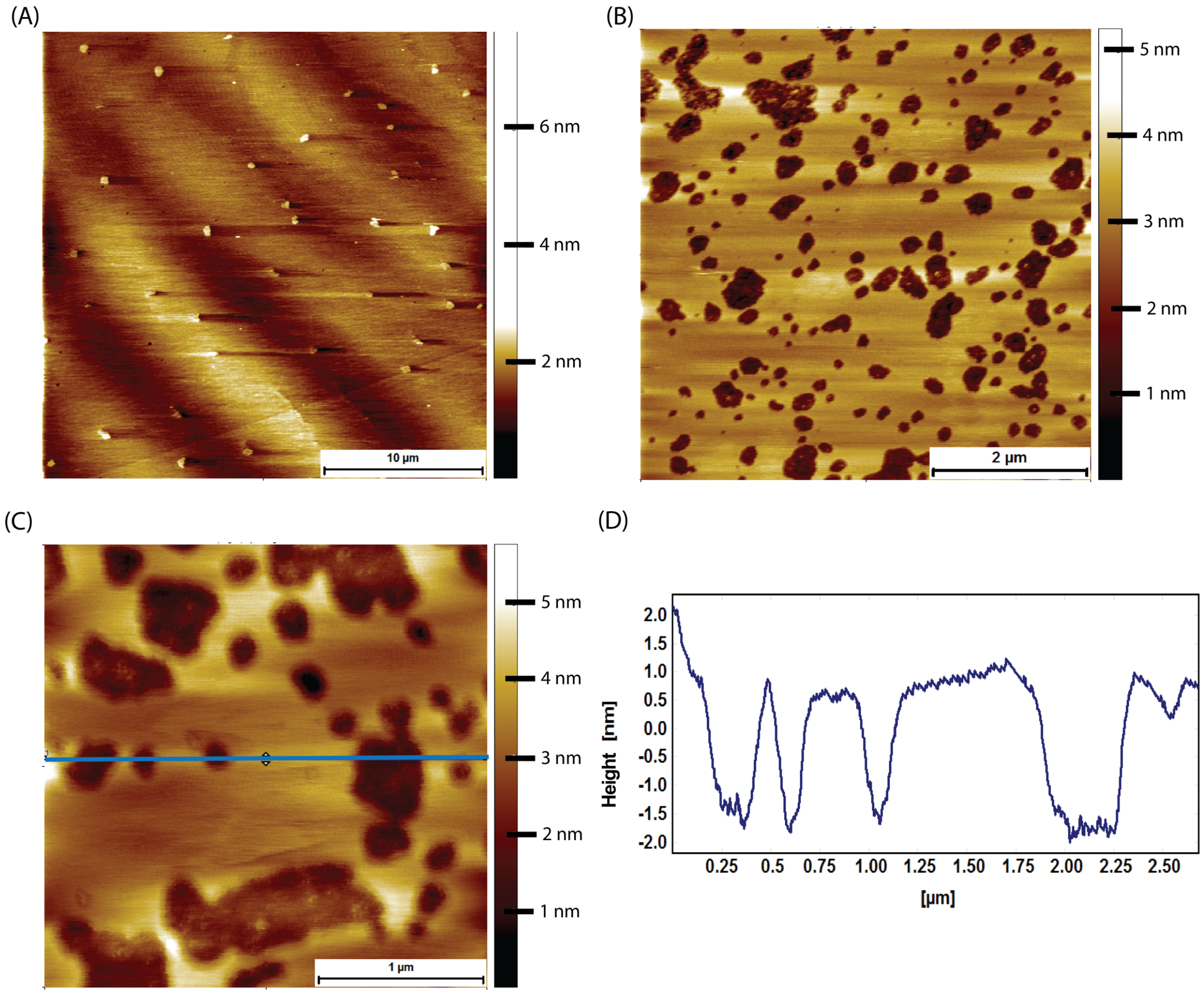

Figure 6 – AFM tapping mode images (in PBS) of mouse brain tissue derived supported lipid bilayers exposed to 8 µM of human amyloid beta for 2 hours. A) Large scale image showing a sparse amount of phase separation prior to amyloid beta adsorption. B) Mouse brain derived lipid bilayer after exposure for 2 hours to 8 µM of human amyloid beta. Pits appear in the membrane, with some structure visible within the pits. C) A close up of the pits showing that they do not break through the entire bilayer, i.e. they are not holes. D) Line profile corresponding to the horizontal blue line in (C). The pits are between 2 and 2.5 nm deep, too shallow to be holes in the bilayer, which is ~3.1 nm thick.

By contrast to what was observed with mouse lipid bilayers, upon exposure of naked mole-rat lipid bilayers to human amyloid beta under the same conditions (8 µM for 2 hours), the naked mole-rat lipid bilayer membrane is completely destroyed (Figure 7). In contrast to the raft covered membrane observed under control conditions (Figure 7A), incubation with amyloid beta results in the presence of only fragments of lipid bilayer (Figure 7B and C), > 5 nm in height relative to the surface (consistent with the thickness of the bilayer rather than the height of a raft, Figure 7D). Membrane fragments after amyloid beta exposure were observed with lipid bilayers formed from lipids extracted from all 3 brains examined.

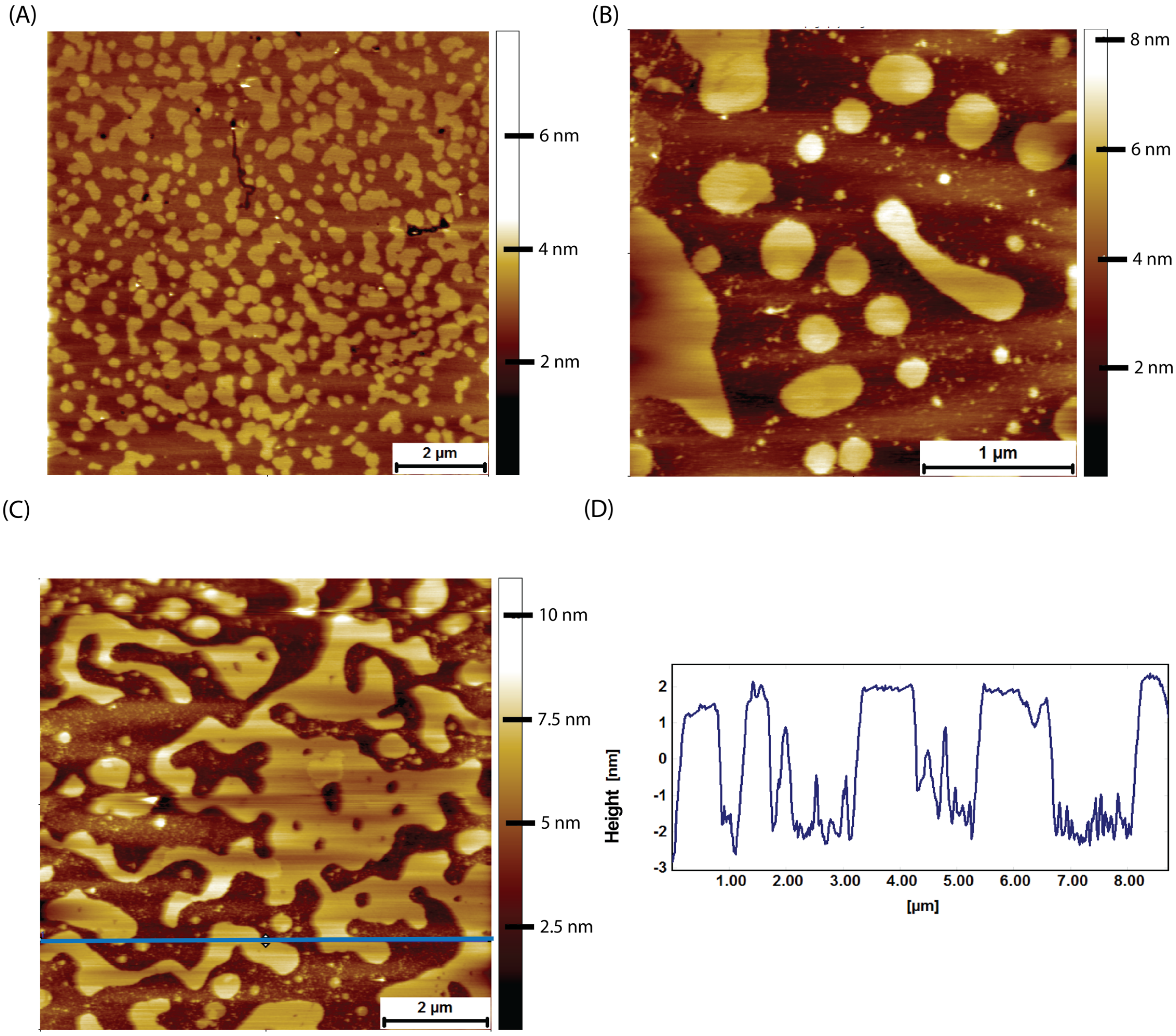

Figure 7 – AFM tapping mode images (in PBS) of naked mole-rat brain derived lipid bilayers exposed to 8 µM of human amyloid beta for 2 hours. A) The naked mole-rat brain derived bilayer before amyloid beta adsorption exhibiting the high degree of phase separation characteristic of naked mole-rat bilayers. B and C) Naked mole-rat brain derived bilayer exposed to 8 µM of human amyloid beta for 3 hours. D) A height profile corresponding to the blue horizontal line in (C), confirming the height of fragments to be of bilayer thickness of 4~nm.

Our lipidomic data show FA and phospholipid trends between mouse and naked mole-rat that are consistent with the previous work of Mitchell et al[18]. However, in addition we have found that cholesterol levels in naked mole-rat brains are significantly higher than in mouse brains and that sphingomyelin levels are significantly lower (and of a shorter chain length). Using AFM, we found that naked mole-rat brain lipids have an extremely high level of phase separation consistent with predictions from the membrane pacemaker theory. Regardless of whether such domains exist in a living cell, the lipids that are able to phase separate could have a role in protection against ageing. Mechanical measurements show that the extra cholesterol results in a stiffer membrane, but surprisingly, these phase-separated and stiffer membranes offer limited protection against amyloid beta induced damage. The susceptibility of naked mole-rat membranes to amyloid beta induced damage was unexpected given the amyloid beta rich environment of the naked mole-rat brains and the lack of overt neurodegeneration in this species[6]. Cell membranes consist of more than just phospholipids, with cytoskeleton, proteins and carbohydrates all contributing to membrane stability. However, the relative difference in response between mouse and naked mole-rat lipids bilayers could be a significant contributor to the amyloid beta response in each species. It should also be noted that the total lipid extract from brain tissue will include not just lipids from cell membranes but also from mitochondria and other organelles. Interestingly, the same susceptibility of naked mole-rat membrane lipids to damage was found when using naked mole-rat amyloid beta peptide (Supplementary material Figures 2 - 5). This is despite the single amino acid difference in sequence whereby Histidine is replaced by Arginine in naked mole rat amyloid beta. We find that naked mole rat amyloid beta readily forms fibres and fibrils (Supplementary Figures 2 and 3) as is the case with human amyloid beta. Again with mouse brain lipids, the naked mole rat amyloid beta sinks into the bilayer leaving the bilayer intact but with easily identifiable “holes” (Supplementary material Figure 4). Upon exposure to naked mole rat amyloid beta the naked mole rat brain derived supported bilayers are broken up into membrane fragments (Supplementary material Figure 5). This consistency in behaviour between human and naked mole rat amyloid beta with respect to membrane interactions strengthens the argument that brain lipid composition may be critical to the physiology of neurodegeneration in naked mole rats.

In summary, naked mole-rat brain lipids have high free cholesterol and low sphingomyelin levels compared to mouse brain lipids and the bilayers formed from naked mole-rat brain lipids are unusual in terms of the extremely high degree of phase separation. By contrast, both mouse (from our data) and pig brain lipids[28] show no/little sign of such two dimensional ordering. The comparative sensitivity of naked mole-rat lipid bilayers to amyloid beta damage suggests that the naked mole-rat has evolved additional neuroprotective mechanisms to support a healthy brain within an amyloid beta rich environment.

Materials and Methods

Animals

Experiments were performed on lipids extracted from C57BL6/J mice and non-breeding naked mole-rats (a mixture of males and females were used of both species, all adults). Mice were conventionally housed with nesting material and a red plastic shelter in temperature-controlled rooms (21 °C) with a 12 h light/dark cycle and access to food and water *ad libitum*. Naked mole-rats were bred in-house and maintained in an inter-connected network of cages in a humidified (~55 %) temperature-controlled room (28 °C) with red lighting (08:00-16:00) and had access to food *ad libitum*. In addition, a heat cable provided extra warmth under 2-3 cages/colony. Mice were humanely killed by cervical dislocation of the neck and cessation of circulation, whereas naked mole-rats were killed by CO_2 exposure followed by decapitation. Experiments were conducted under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 under a Project License (P7EBFC1B1) granted to E. St. J. Smith by the Home Office and approved by the University of Cambridge Animal Welfare Ethical Review Body.

Lipid extraction

Lipid extraction was performed according to a modified chloroform/methanol (2:1, v/v) procedure[29]. Briefly, frozen brain tissue from naked mole-rat or mouse was crushed into small pieces by razor in a petri dish, then the sample was transferred into a 15 mL tube and 2 mL of chloroform/methanol (2:1, v/v) was added; chloroform (Sigma 288306-1L), methanol (Fisher 10675112). The suspension was vortexed and incubated at room temperature with agitation for 20 min. After the incubation, 400 µL of 0.9% NaCl was added to the mixture and was then vortexed and centrifuged at 500 x g for 10 min to separate the organic phase from the aqueous phase. The chloroform (lower) layer was transferred into a new 1.5 mL tube and was centrifuged at 500 x g for 10 min. Finally, the supernatant was collected without touching the pellet and was transferred into a new 1.5 mL tube. The sample was stored at -80 °C until the analysis.

Brain neutral lipids analysis

Internal standards: glyceryl trinonadecanoate, stigmasterol, and cholesteryl heptadecanoate (Sigma) were added to 45 µL of lipid extract. Triglycerides, free cholesterol, and cholesterol esters were analysed with gas-liquid chromatography on a Focus Thermo Electron system equipped with a Zebron-1 Phenomenex fused-silica capillary column (5 m, 0.25 mm i.d., 0.25 mm film thickness). Oven temperature was programmed to increase from 200 to 350 °C at 5 °C/min, and the carrier gas was hydrogen (0.5 bar). Injector and detector temperatures were 315 °C and 345 °C, respectively.

Brain phospholipid, ceramide and sphingolipid analysis

Internal standards (Cer d18:1/15:0, 16 ng; PE 12:0/12:0, 180 ng; PC 13:0/13:0, 16 ng; SM d18:1/12:0, 16 ng; PI 16:0/17:0, 30 ng; PS 12:0/12:0, 156.25 ng; all from Avanti Polar Lipids) were added to 45 µL of lipid extract. Sample solutions were analysed using an Agilent 1290 UPLC system coupled to a G6460 triple quadripole spectrometer (Agilent Technologies). MassHunter software was used for data acquisition and analysis. A Kinetex HILIC column (Phenomenex, 50 × 4.6 mm, 2.6 µm) was used for LC separations. The column temperature was maintained at 40 °C. Mobile phase A was acetonitrile and B was 10 mM ammonium formate in water at pH 3.2. The gradient was as follows: from 10% to 30% B in 10 min, 100% B from 10 to 12 min, and then back to 10% B at 13 min for 1 min to re-equilibrate prior to the next injection. The flow rate of the mobile phase was 0.3 ml/min, and the injection volume was 5 µl. An electrospray source was employed in positive (for Cer, PE, PC, and SM analysis) or negative ion mode (for PI and PS analysis). The collision gas was nitrogen. Needle voltage was set at +4000 V. Several scan modes were used. First, to obtain the naturally different masses of different species, we analysed cell lipid extracts with a precursor ion scan at 184 m/z, 241 m/z, and 264 m/z for PC/SM, PI, and Cer, respectively. We performed a neutral loss scan at 141 and 87 m/z for PE and PS, respectively. The collision energy optimums for Cer, PE, PC, SM, PI, and PS were 25 eV, 20 eV, 30 eV, 25 eV, 45 eV, and 22 eV, respectively. The corresponding SRM transitions were used to quantify different phospholipid species for each class. Two MRM acquisitions were necessary, due to important differences between phospholipid classes. Data were treated with QqQ Quantitative (vB.05.00) and Qualitative analysis software (vB.04.00).

Brain fatty acid analysis

To measure all brain fatty acid methyl ester (FAME) molecular species, internal standard, glyceryl triheptadecanoate (2 µg), was added to 40 µL of lipid extract. The lipid extract was transmethylated with 1 ml BF_3 in methanol (14% solution; Sigma) and 1 ml heptane for 60 min at 80 °C and evaporated to dryness. The FAMEs were extracted with heptane/water (2:1). The organic phase was evaporated to dryness and dissolved in 50 µl ethyl acetate. A sample (1 µl) of total FAME was analysed with gas-liquid chromatography (Clarus 600 Perkin Elmer system, with Famewax RESTEK fused silica capillary columns, 30-m × 0.32-mm i.d., 0.25-µm film thickness). Oven temperature was programmed to increase from 110 °C to 220 °C at a rate of 2 °C/min, and the carrier gas was hydrogen (7.25 psi). Injector and detector temperatures were 225 °C and 245 °C, respectively.

Atomic force microscopy

AFM experiments were performed on an Agilent 5500 with closed loop scanners using a liquid cell for imaging of supported lipid bilayers. All experiments were carried out at room temperature, 20C. Tapping mode imaging was performed using aluminium coated cantilevers (PPP-NCSTR, Apex Probes, UK) and all images were obtained in PBS buffer. Contact mode images were obtained using contact mode aluminium coated silicon cantilevers (PPP-CONTR, Apex Probes, UK) having nominal spring constants of between 0.02 and 0.77 N m^{-1} and typical tip radius of curvatures of less than 7 nm.

Amyloid beta preparation

Beta-amyloid (1-42), human (Anaspec) was reconstituted with 1.0% NH_4OH, diluted in PBS, and then aliquoted before freezing. Before use the aliquot was defrosted and made up to the desired concentration with PBS buffer. This solution was then vortexed for 2 minutes before being added to the supported lipid bilayer. For all experiments 200 µl of 8 µM amyloid beta solution was added to the supported bilayer and left for 2 hr. This was removed by gentle pipetting and the bilayer was rinsed gently three times in PBS buffer to remove any free floating amyloid beta.

Preparation of supported lipid bilayers

2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (DPPC) (Avanti Polar Lipids) were dissolved in chloroform in a 3 to 1 molar ratio and dried under nitrogen. These lipids mixtures were then re-suspended in ultrapure water to a final concentration of 1 mg/ml and intensively vortexed at room temperature. For brain derived lipids the lipids were dried down to form a film and then resuspended in ultrapure water. Uni-lamellar vesicles were formed via ten cycles of freeze-thawing followed by extrusion ten times) through a polycarbonate membrane with pores sizes of 100 nm using a mini-extruder (Avanti Polar Lipids) kept 50 °C on a hotplate. 200 µl of solution containing the extruded vesicles was pipetted onto freshly cleaved muscovite mica and this was then placed on a thin metal disk that itself was placed on a hotplate. The hotplate was heated up to 50 °C and the sample was annealed for 15 minutes. The sample was then loaded into the AFM liquid cell ready for imaging.

Breakthrough force measurements

Indentation measurements were made using contact mode aluminium coated silicon cantilevers (PPP-CONTR, Apex Probes, UK) having nominal spring constants of between 0.02 and 0.77 N m^{-1} and typical tip radius of curvatures of less than 7 nm. The spring constants were calibrated using the equipartition theorem (Thermal K). Actual measured spring constant values were between 0.05 and 0.18 Nm^{-1}. Force volume indentation grids of 256 points (16×16) were taken at no less than three different areas across the bilayer. Indentation rate was 1μm/s which was the optimum rate for measuring breakthrough events. Force distance curves were analyzed using a Scanning Probe Image Processor (Image Metrology, Lyngby, Denmark) and in house developed batch analysis code based on the methodology proposed by Li et al[30].

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Comparisons between-groups were performed by the Mann-Whitney test. Statistical significance was accepted at $P < 0.05$. The heatmaps were performed with the R software (www.r-project.org) with R package, Marray. Ward's algorithm, modified by Murtagh and Legendre, was used as the clustering method.

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