Intercalated MMedSc Final Project Report

α-Dystrobrevin Knockout Mice: A Potential New Model for the Study of Vascular Dementia

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Contents

1. Acknowledgements

This project was funded by a bursary from The Pathological Society, whose financial aid is highly appreciated and acknowledged. I would like to thank Mr Matthew MacGregor Sharp for his expertise and guidance in both electron microscopy imaging techniques and also subsequent analysis. I would also like to thank Professor Roxana Carare for her supervision and guidance throughout this study.

Contributions

Mice used in this study were generated by Darek Górecki *et al.* at the University of Portsmouth. Euthanasia of the mice was carried out in accordance with on-site guidelines. Euthanasia, intracardial perfusion and subsequent preparation for electron microscopy were carried out by Mr Matthew Macgregor Sharp. Imaging of the prepared sections and subsequent segmentation were performed by myself.

2. Introduction

2.1 Vascular Dementia

Vascular dementia (VaD) is the second most common cause of dementia and can arise from a range of vascular pathologies[1]. There is a current lack of mechanistic understanding of the pathophysiology of VaD which has hampered the development of both novel models and effective therapies[2]. Cerebral amyloid angiopathy (CAA), characterised by the deposition of amyloid peptides, such as amyloid-beta (Aβ) in the walls of blood vessels and with no treatment known[3], is a significant contributor to the pathogenesis of VaD. Several animal models of CAA have been produced, however these predominantly work by altering the Aβ peptide produced or by causing its overexpression[4]. Despite this, there is no evidence that increased Aβ is present in sporadic CAA and so failure of Aβ clearance is generally considered the key component of the pathogenesis of CAA[5-7]. The brain lacks conventional lymphatics and so normal clearance of waste products from the brain parenchyma occurs via blood vessels. Elimination takes place via rapid drainage along the (100 – 150 nm thick) basement membranes (BM) of the walls of cerebral capillaries and arteries to cervical lymph nodes, against the direction of blood flow[8]. This process is known as intramural periarterial drainage (IPAD). However, entrapment of insoluble proteins, such as Aβ, in BM disrupts the normal route of elimination of solutes from the brain parenychma[7]. When normal IPAD is disrupted, proteins can accumulate as insoluble deposits within both the walls of capillaries and arteries (as is seen in CAA) and also in the extracellular spaces of the brain parenchyma[7].

Any alterations to the composition or morphology of BM have the possibility to have a knock-on effect on the passage of IPAD and could therefore potentially initiate the deposition of Aβ and begin the pathophysiology of CAA[9]. Changes to the BM, such as duplication, thickening and splitting have been reported in the brains of both aged animals and humans, and furthermore these changes occur to an even greater degree in Alzheimer's disease (AD) demented brains[9]. Additionally, thickening of BM, in humans and rodents, appears to be most common in brain areas that are known to be susceptible to both CAA and AD pathology[9]. The dystrophin-associated protein complex (DAPC) may hold the possibility of providing a new animal model of CAA via producing changes to the BM.

2.2 The Dystrophin-Associated Protein Complex

The DAPC (Figure 1) is a protein complex found in multiple systems of the body[10] and is most well-known for its role in Duchenne muscular dystrophy (DMD), where genetic mutations affecting the dystrophin protein cause destabilisation of the whole protein complex and disrupt its functioning[11]. The major hallmarks of DMD are progressive muscle weakness and skeletal muscle degeneration, however cognitive issues are also seen[12]. Whilst the majority of those affected with DMD are cognitively normal, a meta-analysis carried out by Cotton *et al.*[12] found the full-scale intelligence quotients of those with DMD to be one standard deviation lower than that of the general population, with a mental retardation prevalence of 34.8%. It has been suggested that due to the high concentrations of several components of the DAPC that are found at the glial-vascular interface, there could be a link between the BM and astroctyes, similar to that seen in muscle. This interaction could have a role in stabilizing this interaction[13] and without it could cause changes in the BM, which could consequently affect the passage of IPAD and provides a plausible explanation for the cognitive deficits seen in DMD.

A prominent murine model of DMD is the mdx mouse, which displays cognitive impairments similar to those seen in patients with DMD[14,15] and VaD. The structure of the BM has been shown to be disrupted in mdx mice, becoming thickened and discontinuous[16]. Mdx mice would show promise as a model for CAA, via disruption to BM and hence IPAD, however they have a reduced lifespan[17]. Because increased age is the strongest risk factor for cerebrovascular disease[1], mdx mice are therefore not suitable to model VaD. The DAPC may still hold promise, though, through its other associated protein components.

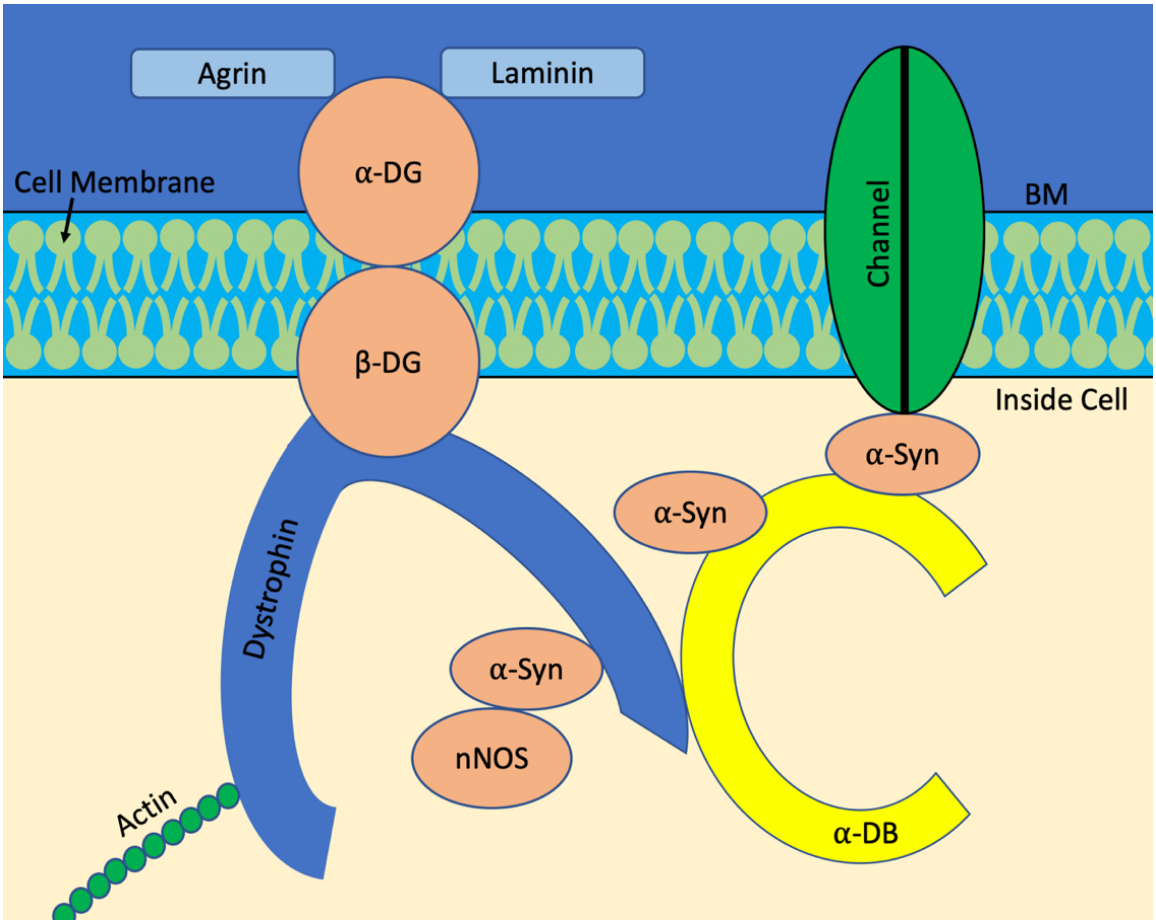

Figure 1. A simplification of the DAPC.

Abbreviations used: α-DG: α-dystroglycan. β -DG: β-dystroglycan. α-Syn: α-syntrophin. α-DB: α-dystrobrevin. nNOS: neuronal nitric oxide synthase. BM: Basement Membrane.

Based on a diagram used by Waite *et al.*[18]

2.3 Alpha-Dystrobrevin: a possible new model of VaD?

α-dystrobrevin (α-DB), shown in yellow in Figure 4, is a protein component of the DAPC found at the cerebrovascular interface. α-DB plays a structural role in stabilising the DAPC and, in skeletal muscle, lack of α-DB compromises the biochemical association between dystrophin, the key protein in the DAPC, and β-dystroglycan[19], which are strongly involved in anchoring the DAPC to BM. This indicates that the α-DB knockout (α-DB$^{-/-}$) mouse, whilst not showing any of the muscular symptoms of DMD, may still show cognitive impairment, similar to that seen in the mdx mouse.

α-DB$^{-/-}$ mice are not reported to have reduced life-spans[20] and furthermore we have initial pilot data showing that IPAD fails in these mice. This provides an exciting prospect for a CAA model of VaD. However, the cerebrovascular BM has not been characterised in these mice. In order to ascertain

the differences between young and aged mice, it is necessary to first understand the exact changes that occur in young α-DB$^{-/-}$ mice, compared to wild-type (α-DB$^{+/+}$).

We hypothesise that in small vessels, composition of the cerebrovascular BM will be affected in α-DB$^{-/-}$ mice, most likely with a thickening of the BM, similar to that seen in mdx mice[16].

3. Methodology

3.1 Animal Acquisition and Subsequent Preparation for Electron Microscopy

10-week-old α-DB$^{-/-}$ mice (n=2) and α-DB$^{+/+}$ littermate controls (n=3) were generated by and obtained from collaborators at the University of Portsmouth. Intracardial perfusion, and later sectioning, were carried out by colleagues following our groups published protocols[21]. These were processed for electron microscopy following protocols optimised by our group[22], trimmed into 80 nm sections using an Ultracut E microtome and then placed onto copper grids, before finally being counter-stained with lead citrate.

3.2 Imaging

Grids were visualized with a FEI Tecnai T12 transmission electron microscope (TEM) employing a Morada G2 camera digital camera and Radius image capture software (EMSIS, Münster, Germany). 40 high resolution micrographs of small vessels were taken digitally per mouse (20 per region).

3.3 Data Analysis

Quantitative analysis was undertaken using Adobe Photoshop CS6, a software often used to prepare and measure digital images, which is excellent at aiding the separation of key features of interest from background detail[23]. As a range of vessel sizes were imaged, the collective term of “intramural cell” (IMC) was given to both smooth muscle cells and pericytes within the BM.

Photoshop was used to segment out the different vessel wall components (endothelium, IMCs and BM) and then to calculate the percentage surface areas of each component. Percentages surface area, as opposed to true surface area, was used as a measurement in order to remove any skew that could be introduced by different vessel sizes. This process of extracting quantitative data is outlined in greater detail in Figure 2 and visualised in Figure 3.

Step	Process
Step 1	Image uploaded to Adobe Photoshop CS6 for segmentation. The following vessel components were identified: Lumen, Endothelium, Endothelial Nucleus (if present), Intramural Cell (if present), Basement Membrane. This process can be seen in Figure 3.
Step 2	After identification, the anatomical lines that separate each component were traced with different colours, before application of a colour overlay was employed. This process and the colour scheme employed can be seen in Figure 3.
Step 3	Image size variables, such as pixel length and magnification, were used to calculate the pixel:µm ratio. This ratio was input back into Adobe Photoshop and the area of each component of the vessel (in μm^2) was extracted.
Step 4	Calculation of the percentages of the vessel wall taken up by the endothelium, IMCs and BM.

Figure 2. A table outlining the process of quantitative data extraction from TEM images.

3.4 Statistical Analysis

Statistical analysis was performed using SPSS software (Version 25) with Univariate Analysis of Variance (two-way ANOVA test), adjusting for multiple sample points (40 per mouse) with significance set at $P < 0.05$.

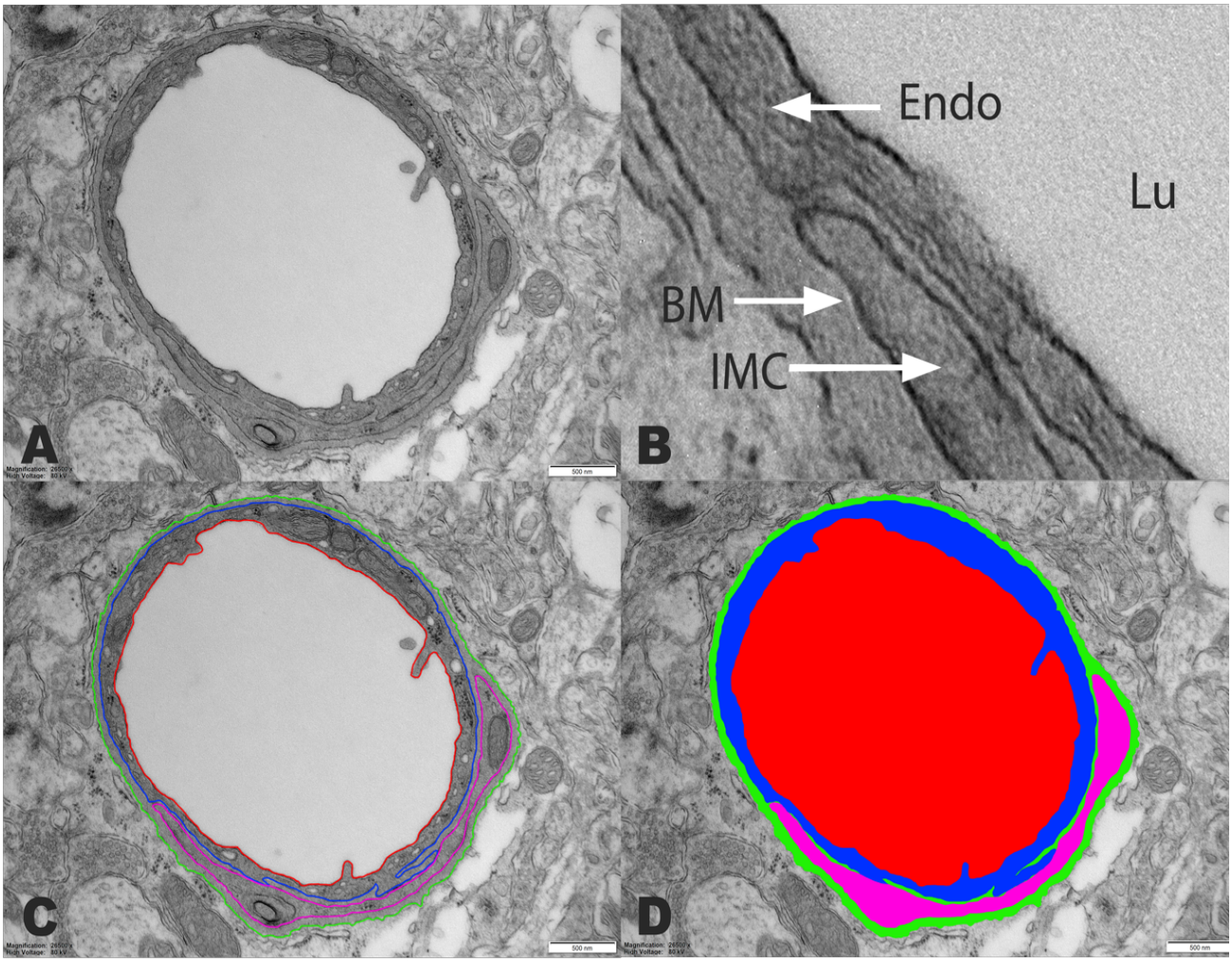

Figure 3. A visual representation of steps 1 and 2 outlined in Figure 2. In step 1, an image is uploaded to photoshop (A) and the different vessel components are identified (B). In step 2, the different components are outlined (C), with the colour scheme of lumen (Lu) in red, endothelium (Endo) in blue, intramural cell (IMC) in pink and basement membrane (BM) in green. Colour overlay is then employed in order to allow quantitative assessment of different vessel components (D).

4. Results

4.1 There is no difference between the Grey and White Matter in Wild Type Mice

Small vessels from grey matter (n=60) and white matter (n=60) of the α-DB$^{+/+}$ control mice were compared in order to determine if there was any difference in the percentage surface areas of their vessel wall components. Percentage surface areas were: endothelium (55.76% vs 54.94%), basement membrane (31.85% vs 31.14%) and intramural cells (12.39% vs 13.92%) (Figure 5). A Univariate Analysis of Variance showed no significant difference in the surface area occupied by any vessel wall components (Figure 4).

	Source	Sum of Squares	df	Mean Square	F	Sig.
Endothelium	Contrast	13.421	1	13.421	0.188	0.666
	Error	4212.275	59	71.394		
BM	Contrast	10.031	1	10.031	0.411	0.524
	Error	1441.034	59	24.424		
IMC	Contrast	46.658	1	46.658	1.007	0.320
	Error	2734.399	59	46.346		

Figure 4. A table showing the results of the two-way ANOVA test for the grey and white matter of the α-DB$^{+/+}$ mice. Results are shown to three decimal places. Significance is set at $P < 0.05$, with significant values displayed in green and non-significant values in red.

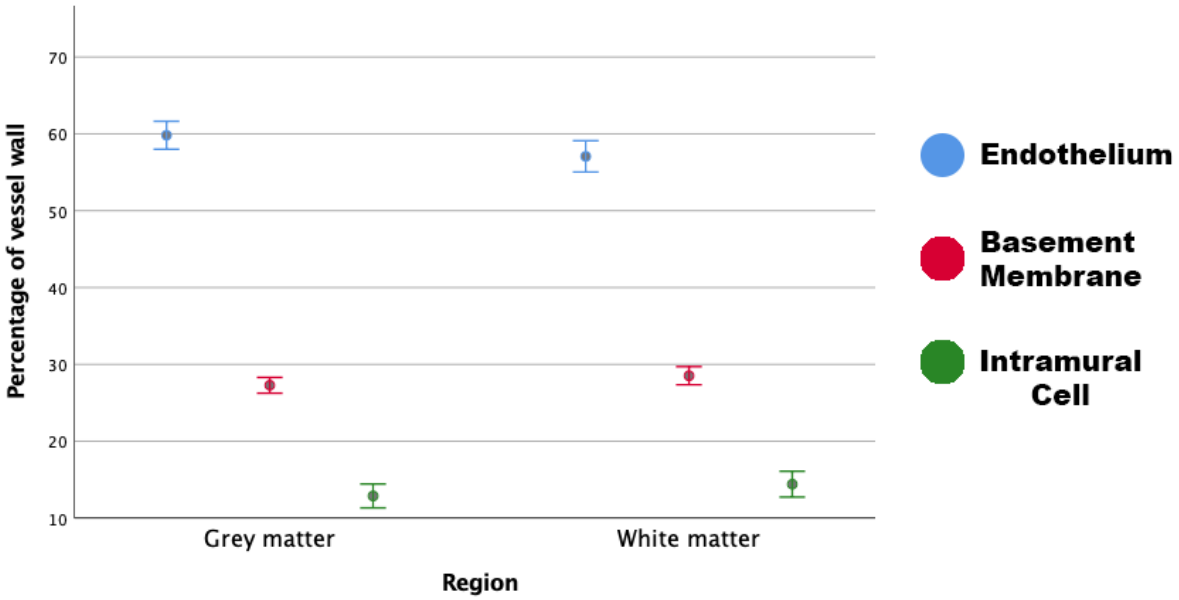

Figure 5. A graph comparing the percentage of the vessel wall taken up by the different vessel wall components for the grey and white matter of α-DB$^{+/+}$ mice. Error bars represent a 95% confidence interval.

4.2 There is no difference between the Grey and White Matter in Mice deficient for α-Dystrobrevin

Small vessels from grey matter (n=40) and white matter (n=40) of the α-DB $^{-/-}$ mice were compared in order to determine if there was any difference in the percentage surface areas of their vessel wall components. Percentage surface areas were: endothelium (59.82% vs 57.07%), basement membrane (27.28% vs 28.52%) and intramural cells (12.88% vs 14.41%) (Figure 7). A Univariate Analysis of Variance showed no significant difference in the surface area occupied by any vessel wall components (Figure 6).

	Source	Sum of Squares	df	Mean Square	F	Sig.
Endothelium	Contrast	226.954	1	226.954	3.902	0.51
	Error	5758.316	99	58.165		
BM	Contrast	46.322	1	46.322	2.355	0.128
	Error	1947.348	99	19.670		
IMC	Contrast	70.155	1	70.155	1.771	0.186
	Error	3922.280	99	39.619		

Figure 6. A table showing the results of the two-way ANOVA test for the grey and white matter of the α-DB$^{-/-}$ mice. Results are shown to three decimal places. Significance is set at $P < 0.05$, with significant values displayed in green and non-significant values in red.

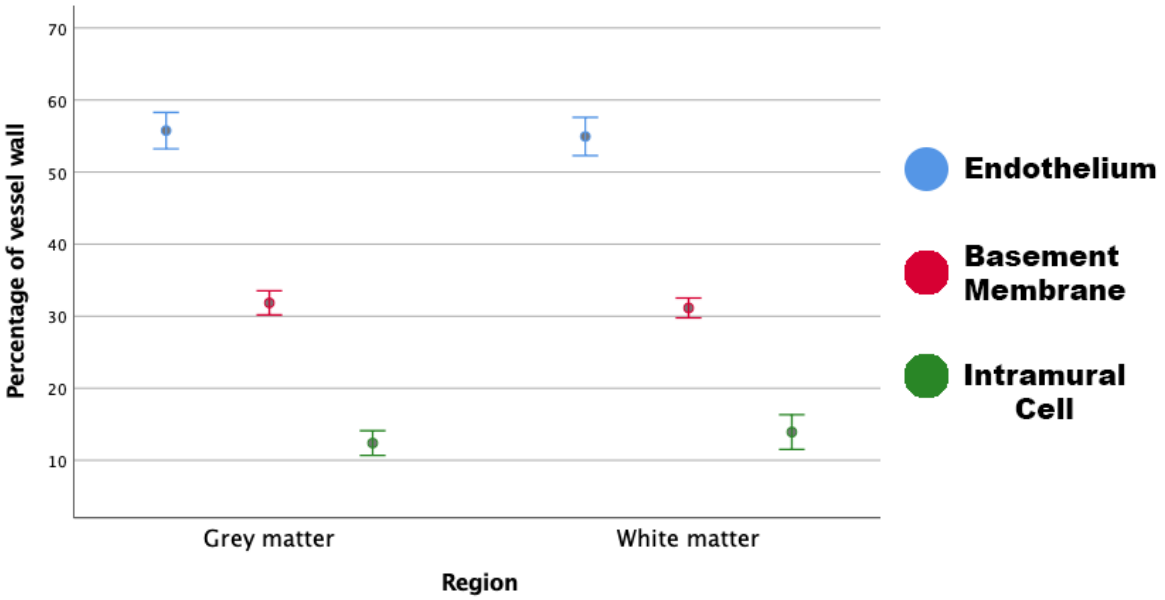

Figure 7. A graph comparing the percentage of the vessel wall taken up by the different vessel wall components for the grey and white matter of α-DB$^{-/-}$ mice. Error bars represent a 95% confidence interval.

4.3 Mice deficient for α-Dystrobrevin have a thinner Endothelium and a thicker Basement Membrane in the Grey Matter

Small vessels from the grey matter of α-DB$^{-/-}$ mice (n=40) and α-DB$^{+/+}$ mice (n=60) were compared in order to determine if there was any difference in the percentage surface areas of their vessel wall components. Percentage surface areas were: endothelium (55.76% vs 59.82%), basement membrane (31.85% vs 27.28%) and intramural cells (12.39% vs 12.88%) (Figure 9). A Univariate Analysis of Variance showed a significant difference in the surface area occupied by the endothelium ($p = 0.013$) and the basement membrane ($p < 0.001$) (Figure 8).

	Source	Sum of Squares	df	Mean Square	F	Sig.
Endothelium	Contrast	395.004	1	395.004	6.393	0.013
	Error	4880.874	79	61.783		
BM	Contrast	500.961	1	500.961	23.492	>0.001
	Error	1684.678	79	21.325		
IMC	Contrast	5.774	1	5.774	0.153	0.696
	Error	2976.088	79	37.672		

Figure 8. A table showing the results of the two-way ANOVA test for the grey matter of both the α-DB$^{-/-}$ and α-DB$^{+/+}$ mice. Results are shown to three decimal places. Significance is set at $P < 0.05$, with significant values displayed in green and non-significant values in red.

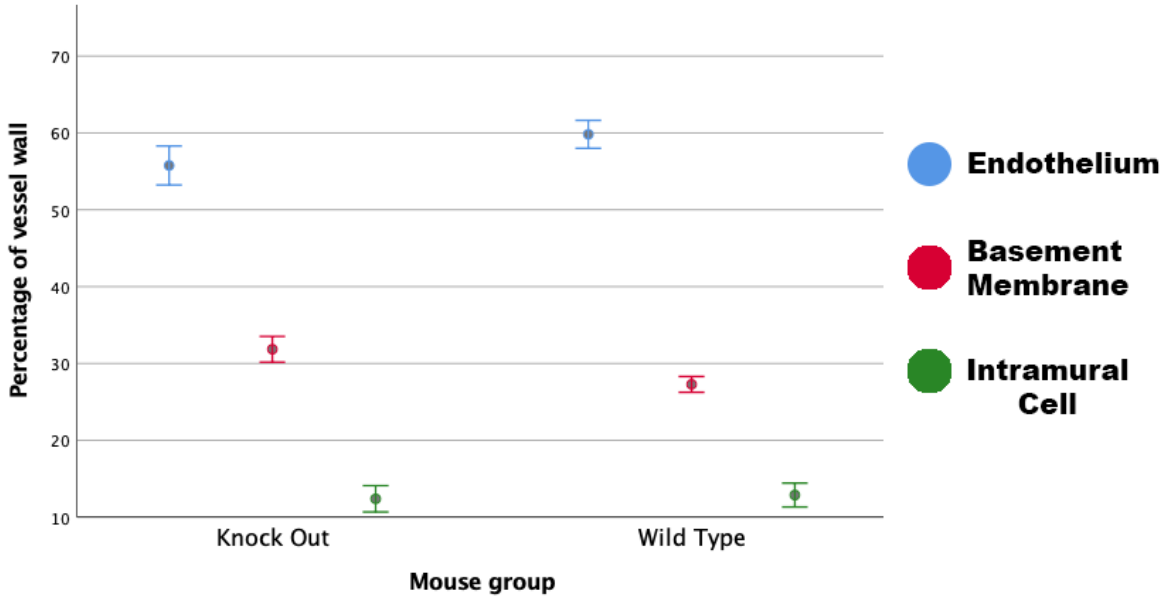

Figure 9. A graph comparing the percentage of the vessel wall taken up by the different vessel wall components for the grey matter of α-DB$^{-/-}$ and α-DB$^{+/+}$ mice. Error bars represent a 95% confidence interval.

4.4 Mice deficient for α-Dystrebrevin have a thicker Basement Membrane in the White Matter

Small vessels from the white matter of α-DB$^{-/-}$ mice (n=40) and α-DB$^{+/+}$ mice (n=60) were compared in order to determine if there was any difference in the percentage surface areas of their vessel wall components. Percentage surface areas were: endothelium (54.94% vs 57.07%), basement membrane (31.14% vs 28.52%) and intramural cells (13.92% vs 14.41%) (Figure 11). A Univariate Analysis of Variance showed a significant difference in the surface area occupied by the endothelium ($p = 0.006$) (Figure 10).

	Source	Sum of Squares	df	Mean Square	F	Sig.
Endothelium	Contrast	108.437	1	108.437	1.592	0.211
	Error	5380.092	79	68.102		
BM	Contrast	164.486	1	164.486	8.024	0.006
	Error	1619.409	79	20.499		
IMC	Contrast	5.817	1	5.817	0.112	0.739
	Error	4107.959	79	51.999		

Figure 10. A table showing the results of the two-way ANOVA test for the white matter of both the α-DB$^{-/-}$ and α-DB$^{+/+}$ mice. Results are shown to three decimal places. Significance is set at $P < 0.05$, with significant values displayed in green and non-significant values in red.

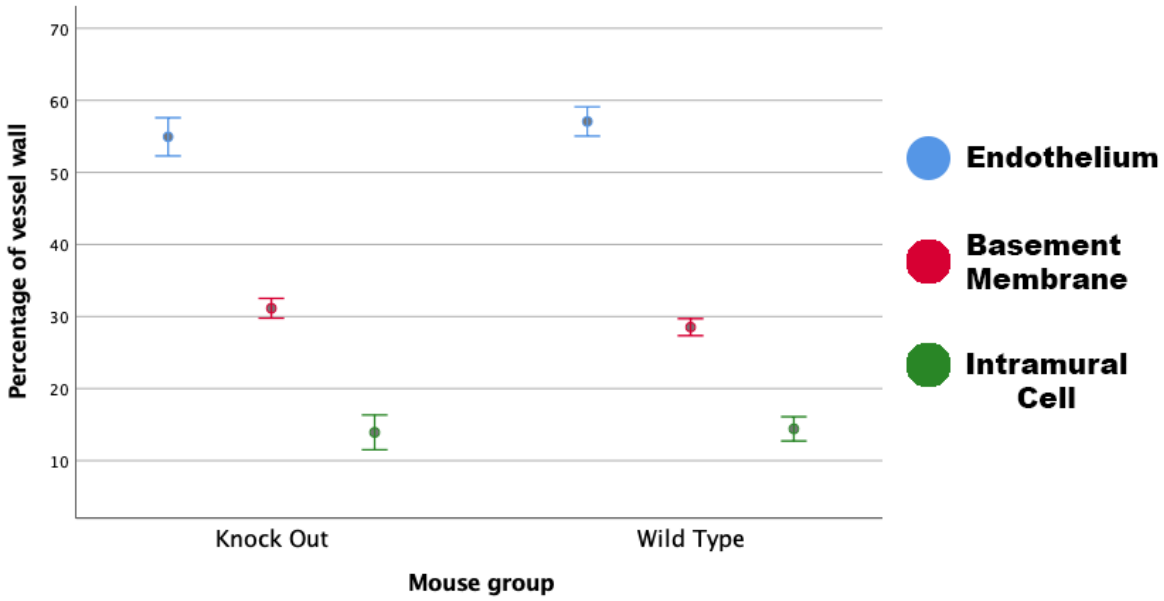

Figure 11. A graph comparing the percentage of the vessel wall taken up by the different vessel wall components for the white matter of α-DB$^{-/-}$ and α-DB$^{+/+}$ mice. Error bars represent a 95% confidence interval.

5. Discussion

The objective of this study was to ascertain whether α-DB $^{-/-}$ mice hold promise as a possible novel animal model of CAA and VaD. This was undertaken via investigating the ultrastructural differences in the cerebrovasculature of the grey and white matter in α-DB $^{-/-}$ mice. TEM was employed to assess the composition of blood vessels through comparison of percentage surface areas taken up by different vessel wall components. We hypothesised that composition of the BM would be disrupted in α-DB$^{-/-}$, most likely with a thickening of the BM – as is seen in mdx mice[16], another mouse model with DAPC alteration.

5.1 A First Step in the Development of a new model of Vascular Dementia

In our analysis, the BM of the grey and white matter was thicker in mice deficient for α-DB. These alterations to the BM could possibly have been caused by disruption to the astrocyte-BM anchoring system, which we believe is in part regulated by the DAPC. This provides an initial exciting prospect for mice lacking α-DB as a model for CAA. Changes to BM have been shown to proceed deposition of Aβ in the vessel walls[24] and therefore this is an important aspect to reproduce as both mechanistic understanding and therapeutics rely on models that replicate the true pathogenesis of the disease. It is especially of note that the mice used in this study were only aged to 10 weeks, yet still displayed significant changes to the BM. It has been shown that cerebrovascular BM thickening occurs with ageing and this also especially happens in the brains of AD patients[25]. Furthermore, mice show thickening of their cerebrovascular BM as they age[26]. This demands further research using aged mice to assess how the BM of mice deficient for α-DB change as they age. Additionally, investigations into whether IPAD is affected in α-DB$^{-/-}$ mice, if there are pathological changes in their brains and finally to study if they display any cognitive deficits.

6. Conclusion

In this study, we demonstrated the ultrastructural changes that occur in α-DB$^{-/-}$ mice, with particular interest into any changes seen in the BM, which are the conduits for IPAD and hence implicated in the pathogenesis of CAA and VaD. We found that in α-DB$^{-/-}$ mice, there is a resultant thickening of the BM in both the grey and white matter. Whereas the findings demonstrated in this study do point to the exciting prospect that mice deficient for α-DB could be a possible model for VaD, they are only a first step and further study is required to validate them as a good model.

7. References

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