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A cross-brain-regions study of *ANK1* DNA methylation in different neurodegenerative diseases.

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ABSTRACT

Recent epigenome-wide association studies in Alzheimer's disease have highlighted consistent robust neuropathology-associated DNA hypermethylation of the Ankyrin 1 (*ANK1*) gene in the cortex. The extent to which altered *ANK1* DNA methylation is also associated with other neurodegenerative diseases is not currently known. In the current study, we used bisulfite pyrosequencing to quantify DNA methylation across eight CpG sites within a 118bp region of the *ANK1* gene across multiple brain regions in Alzheimer's disease, Vascular dementia, Dementia with Lewy bodies, Huntington's disease and Parkinson's disease. We demonstrate disease-associated *ANK1* hypermethylation in the entorhinal cortex in Alzheimer's disease, Huntington's disease and Parkinson's disease, whilst in donors with Vascular dementia and Dementia with Lewy bodies we observed elevated *ANK1* DNA methylation only in individuals with co-existing Alzheimer's disease pathology. We did not observe any disease-associated differential *ANK1* DNA methylation in the striatum in Huntington's disease, or the substantia nigra in Parkinson's disease. Our data suggests that *ANK1* is characterized by region and disease-specific differential DNA methylation in multiple neurodegenerative diseases.

Keywords:

Alzheimer's disease (AD); Ankyrin 1 (ANK1); Brain; Dementia with Lewy Bodies (DLB); DNA methylation (5-methylcytosine – 5mC); Epigenetics; Huntington's disease (HD); Parkinson's disease (PD); Vascular dementia (VaD)

1. INTRODUCTION

Dementia encompasses a group of chronic neurodegenerative diseases that affected an estimated 46.8 million people worldwide in 2015 (Wimo et al., 2017), of which Alzheimer's disease (AD) accounts for ~60% of cases. The etiology of AD has been hypothesized to involve epigenetic mechanisms (Lunnon and Mill, 2013). In 2014 two epigenome-wide association studies (EWAS) of AD identified significant hypermethylation of CpG sites in the Ankyrin 1 (*ANK1*) gene associated with neuropathology in AD cortex (De Jager et al., 2014; Lunnon et al., 2014), which has been replicated in multiple independent study cohorts (Smith, 2017). Subsequently, a genome wide association study (GWAS) of a Han Chinese population identified a single-nucleotide polymorphism (SNP) in *ANK1* associated with an increased susceptibility for developing AD (Chi et al., 2015). *ANK1* links integral membrane proteins to the underlying spectrin-actin cytoskeleton and plays a key role in cell motility, activation, proliferation, contact, and maintenance of specialized membrane domains (Yang et al., 2011). There is now increasing interest in understanding the role of epigenetic changes in *ANK1* in the development and progression of AD. One important question to be addressed is whether *ANK1* hypermethylation is specific to AD, or observed in other neurodegenerative disorders. Although AD accounts for ~60% of dementia cases, many other dementias share common symptoms and/or pathological hallmarks with AD.

This study aimed to quantify DNA methylation levels across a 118bp region of *ANK1*, previously associated with AD, in a number of different neurodegenerative diseases. Using bisulfite pyrosequencing we assessed *ANK1* DNA methylation in brain samples from donors with AD, Dementia with Lewy bodies (DLB), Vascular dementia (VaD), Huntington's disease (HD), Parkinson's disease (PD) and non-demented

elderly controls, across a number of different brain regions that are characterized by disease-specific pathology.

2. MATERIALS AND METHODS

2.1. Subjects and Samples

Post-mortem brain tissue was obtained from six different UK brain banks (the South West Dementia Brain Bank (SWDBB), the London Neurodegenerative Disease Brain Bank (LNDBB) the Manchester Brain Bank, the Oxford Brain Bank, the Cambridge Brain Bank and the Newcastle Brain Bank). In total, tissue was obtained from 60 AD (Braak V-VI), 119 DLB, 27 VaD, 22 HD, 36 PD and 105 elderly non-demented control subjects (Braak 0-II). A subset of DLB (N = 39) and VaD (N = 5) cases also had co-existing AD pathology. For each disease we analyzed the entorhinal cortex (EC), superior temporal gyrus (STG) and cerebellum (CER). For HD cases we also analyzed the striatum (STR) as this is primarily affected in disease (Reiner et al., 2011), whilst for PD cases we analyzed the STR and substantia nigra (SN) as these are regions of pathology in this disease (Fearnley and Lees, 1991). For control samples we analyzed all five brain regions. For a small number of donors tissue was not available from all brain regions. Genomic DNA was isolated from ~100mg of each dissected brain region using a standard phenol-chloroform extraction method, and tested for degradation and purity prior to analysis as previously described (Smith et al., 2016). Demographic information for samples can be found in **Supplementary Table 1**.

2.2. ANK1 bisulfite pyrosequencing

Bisulfite pyrosequencing was used to quantify DNA methylation across eight individual CpG sites in the *ANK1* gene, spanning from 41519302 to 41519420 within

chromosome 8 (hg19). Bisulfite conversion was performed using the Bisulfite-Gold kit (Zymo research, USA). A single amplicon (246bp) was generated using primers designed using the PyroMark Assay Design software 2.0 (Qiagen, UK) as previously described (Lunnon et al., 2014). Pyrosequencing was performed using two sequencing primers to maximize coverage across eight CpG sites. DNA methylation was quantified using the Pyromark Q24 system (Qiagen, UK) following the manufacturer's standard instructions and the Pyro Q24 CpG 2.0.6 software.

2.3. Data Analysis

All computations and statistical analyses were performed using R 3.3.2 (R Development Core Team, 2012). A linear regression analysis was performed, controlling for the effects of age, gender and batch effects, comparing control samples with samples affected by each neurodegenerative disease. For the VaD and DLB samples we also performed a second analysis to investigate whether co-existing AD pathology influenced the results by comparing individuals with and without co-existing AD pathology to control samples, again accounting for the effects of age, gender and batch. Our analyses examined DNA methylation differences at (a) individual CpG sites and (b) averaged across the amplicon. We used a paired two-tailed t-test to compare adjusted DNA methylation differences in disease across brain regions.

3. RESULTS

3.1. AD-associated ANK1 DNA hypermethylation is seen across all tissues analyzed

First, we sought to replicate previous findings of *ANK1* DNA hypermethylation in AD. Across the 118bp region, we observed significantly increased levels in AD cases

compared to controls in all eight *ANK1* CpG sites in the EC (**Figure 1A**) and seven *ANK1* CpG sites in the STG (**Figure 1B**). Both the EC and STG exhibit a high degree of AD pathology, even in the earliest stages of disease, with the EC being the starting point of AD pathology in the cortex with pathology seen here in Braak stage II (Braak and Braak, 1991). Conversely, the CER remains free of AD pathology until the very last stages of the disease, although even then this is limited to amyloid beta (Aβ) plaques with an absence of neurofibrillary tangles (NFTs) of hyperphosphorylated tau (Braak et al., 1989). Of note, we observed significant *ANK1* DNA hypermethylation at five *ANK1* CpG sites in the CER (**Figure 1C**). This is the first time *ANK1* DNA methylation changes have been reported in the CER. Interestingly, two of the loci that did not display AD-associated *ANK1* hypermethylation in the CER were chr8:41519308 and chr8:41519399, the two sites included on the Illumina 450K array used in previous EWAS analyses of AD which did not identify *ANK1* hypermethylation in AD in the CER (Lunnon et al., 2014). Average DNA methylation across the amplicon region was significantly elevated in AD in the EC (P = 1.29 x 10^{-07}), STG (P = 2.39 x 10^{-03}) and CER (P = 7.81 x 10^{-03}) (**Figure 1D**). *ANK1* DNA methylation differences between cases and controls at both individual sites and across the amplicon were lower in the CER compared to other tissues tested (**Supplementary Table 2**), with a significantly greater DNA methylation difference between cases and controls in the EC (amplicon average corrected DNA methylation difference (Δ) = 4.53%) compared to both the STG (amplicon average Δ = 2.84%; P = 7.98 x 10^{-4}) and the CER (amplicon average Δ = 1.17%, P = 2.55 x 10^{-4}). Interestingly, this pattern of change matches the spread of AD pathology throughout the brain.

3.2. ANK1 DNA hypermethylation in the EC is only observed in DLB cases with co-existing AD pathology

DLB is the third most common cause of dementia with the age of onset ranging from 50 to 83 years (McKeith, 2002). The pathology of DLB shares similarities to AD, with the presence of immune regulation and microglial activation being consistent between diseases (Mackenzie, 2000). However, the presence of Lewy bodies within the brain makes DLB considerably more comparable to PD (McKeith, 2002). In fact, PD dementia is thought to be biologically identical to DLB, only differing in the order in which the motor or cognitive symptoms occur (Dodel et al., 2008). Interestingly we observed significant hypermethylation of *ANK1* in DLB cases compared to controls in the EC (**Figure 2A**) at four of the eight *ANK1* CpG sites **(Supplementary Table 3)**. We saw no difference between DLB and control samples in either the STG (**Supplementary Figure 1A**) or the CER (**Supplementary Figure 1B**) at any of the eight *ANK1* CpG sites. Across the *ANK1* amplicon we observed significant DLB-associated hypermethylation in the EC ($P = 0.0244$), but not in the STG or CER ($P > 0.05$) (**Figure 2B**). It is widely reported that DLB and AD frequently co-occur (Rosenberg et al., 2001); we were therefore interested to investigate whether we still observed DLB-associated DNA hypermethylation in the EC when we controlled for co-existing AD pathology. We found no significant changes in *ANK1* DNA methylation in individuals with "pure" DLB compared to controls in the EC (**Figure 2C**), STG (**Supplementary Figure 1C**) and CER (**Supplementary Figure 1D**). However, we did observe significant hypermethylation in DLB cases with co-existing AD pathology compared to controls at seven of the eight *ANK1* CpG sites in the EC **(Figure 2C)** and two sites in the STG **(Supplementary Figure 1C),** with no difference in the CER (**Supplementary Figure 1D**). When we looked across the whole 118bp region, we saw increased *ANK1* DNA methylation in the EC in individuals with co-existing AD pathology ($P = 1.45 \times 10^{-03}$) **(Figure 2D**), suggesting that the *ANK1* hypermethylation seen in some individuals with DLB is primarily driven by AD pathology.

3.3. ANK1 hypermethylation is seen in the EC only in VaD individuals with co-existing AD pathology

Characterized by the loss of neurological function due to ischemic events, the risk of developing VaD is closely linked to vascular health (Román et al., 1993). We observed increased DNA methylation in individuals with VaD at none of the ANK1 CpG sites in the EC (**Figure 3A**), only one site in the STG (**Supplementary Figure 2A**) and no sites in the CER (**Supplementary Figure 2B**) (**Supplementary Table 4**), with no difference across the amplicon in any of the brain regions tested ($P > 0.05$) (**Figure 3B**). Because VaD also often co-occurs with AD we next examined whether stratifying cases by the presence of AD pathology altered these findings. Interestingly, we saw disease-associated hypermethylation in the EC at five of the eight ANK1 CpG sites only in individuals with co-existing AD pathology (**Figure 3C**), whilst we saw disease-associated hypomethylation at one site in the STG (**Supplementary Figure 2C)** in individuals with "pure" VaD, and no disease-associated changes in the CER (**Supplementary Figure 2D**). When we looked across the 118bp region we only saw significant *ANK1* hypermethylation in individuals with VaD and co-existing AD pathology compared to controls in the EC ($P = 0.0163$) **(Figure 3D)**. It is worth noting that our cohort only had a small number of VaD cases with co-existing AD pathology (N=5).

3.4. ANK1 DNA hypermethylation in the EC is seen in both HD and PD.

HD is characterized by a trinucleotide repeat in the huntingtin gene (*HTT*). The abundance of the repeat is proportional to the level of protein misfolding and downstream cytosolic accumulation, leading to neuronal cell death and the symptoms of HD (Walker, 2007). *ANK1* DNA hypermethylation was seen at four of the eight CpG sites in the EC in HD **(Figure 4A**). However, no differential DNA methylation was seen in the other brain regions tested (**Supplementary Table 5**), including the STG (**Supplementary Figure 3A**), the CER (**Supplementary Figure**

3B) and the STR (**Supplementary Figure 3C**), a region that forms part of the basal ganglia, known to be the first brain region to be adversely affected by HD pathology (Walker, 2007). Averaging across the region again highlighted significant hypermethylation in the EC (P = 6.68 x 10^{-3}), with no significant change in any other tissue **(Figure 4B)**.

A similar pattern of *ANK1* hypermethylation was observed in PD; two of the eight CpG sites were characterized by significant hypermethylation in the EC (**Figure 4C**), with no differences in DNA methylation in any of the other brain regions tested (**Supplementary Table 6**). This included the STG (**Supplementary Figure 4A**), the CER (**Supplementary Figure 4B**), the STR (**Supplementary Figure 4C**) and the SN (**Supplementary Figure 4D**), with the SN representing the brain region that has the highest levels of pathology in PD (Fearnley and Lees, 1991). Across the 118bp amplicon we saw no change in DNA methylation in any of the five brain regions (**Figure 4D**).

4. DISCUSSION

This is the first study to assess brain *ANK1* DNA methylation changes across multiple neurodegenerative diseases. We identified significant DNA methylation changes in the EC in multiple diseases, including AD, HD and PD, with significant DNA hypermethylation across the amplicon in AD and HD. Interestingly, we also observed significant hypermethylation of *ANK1* in the EC in both DLB and VaD at several individual CpG sites and across the amplicon, but only in donors with co-existing AD pathology. This suggests that *ANK1* DNA hypermethylation in the EC is specific to some neurodegenerative diseases (AD, HD and to some extent PD), and not observed in other forms of neuropathology (VaD and DLB). Although it is possible

that the observed changes in *ANK1* could reflect a common feature of neurodegenerative diseases, such as neuroinflammation, it is worth noting that we did not observe ANK1 DNA hypermethylation in all diseases, for example we did not see any hypermethylation in individuals with “pure” DLB or VaD. These diseases are also characterised by neuroinflammation, so this suggests that the observed hypermethylation does not simply reflect a common hallmark of all neurodegenerative diseases such as microgliosis. We have previously reported that *ANK1* is not hypermethylated in the CER in AD at two sites interrogated by the Illumina 450K array (chr8:41519308 and chr8:41519399). In the current study we again demonstrate that these two loci are not significantly differentially methylated in AD; however, we do highlight AD-associated DNA hypermethylation at five adjacent CpG sites and averaged across the 118bp amplicon in the CER. We did not see any *ANK1* DNA methylation changes in the CER in any of the other neurodegenerative diseases, including those with co-existing AD pathology. Reflecting our previous findings, we found that DNA methylation differences in AD are greatest in the EC, an area with high levels of neuropathology and lowest in the CER, the region with the least neuropathology. Interestingly, although we observed disease-associated *ANK1* hypermethylation in the EC at six individual sites and across the region in HD and at two individual sites in PD, we did not see *ANK1* DNA methylation changes in these diseases in their regions of primary neuropathology, namely the STR and SN, respectively. This suggests that *ANK1* hypermethylation in neurodegenerative disease is not necessarily specific to regions of primary neuropathology, but may instead be specific to particular cell types affected in only specific diseases, such as those in the EC, which are not present in the STR and SN. *ANK1* encodes for numerous isoforms with their own tissue-specific enhancers. Although the precise function of most *ANK1* isoforms is not known, different isoforms have been identified in the brain, blood and muscle (Gallagher et al., 1997). It would be of interest to examine expression levels of different *ANK1* transcript variants, to facilitate the

interpretation of the DNA methylation differences we observe. Mastroeni *et al*., recently showed a four-fold increase in *ANK1* mRNA expression in microglia from AD brain tissue, but not in neurons or astrocytes from the same individuals, suggesting an immune based function for ANK1 in the human brain (Mastroeni et al., 2017). One potential caveat of our study is that we have analyzed "bulk" tissue, and we cannot determine which cell type(s) are driving the DNA hypermethylation seen in *ANK1* in disease.

Although the focus of our study was on investigating DNA methylation changes in disease, bisulfite pyrosequencing actually generates a summative measurement of both DNA methylation and DNA hydroxymethylation. DNA methylation is generally associated with gene silencing, particularly when residing in the promoters of constitutively expressed housekeeping genes (Jones, 2012), whilst DNA hydroxymethylation has been shown to be enriched in gene bodies (Lunnon et al., 2016) and to be found at (relatively) high levels in the brain (Khare et al., 2012; Szulwach et al., 2011). We have recently shown that *ANK1* DNA hypermethylation across the 118bp amplicon in AD is actually underestimated when using bisulfite data, as it is confounded by significant DNA hypohydroxymethylation at some loci in the amplicon (Smith et al., Under Review). Another caveat to our study is that we have only analyzed DNA methylation across eight CpG sites in a 118bp region of the *ANK1* gene and thus future studies should aim to further quantify changes in DNA methylation across the entire 244kb gene.

5. CONCLUSIONS

Our study has demonstrated disease-associated *ANK1* hypermethylation in the EC at specific CpG sites in AD, HD and PD and across the region in AD and HD. In donors with DLB and VaD we only observed increased *ANK1* DNA methylation in the EC in

individuals with co-existing AD pathology. The CER showed disease-associated hypermethylation at specific CpG loci and across the region in AD, but not in any of the other neurodegenerative diseases tested. We saw no disease-associated differential *ANK1* DNA methylation in the STR in HD or PD, or the SN in PD. This suggests that *ANK1* is characterized by brain region and disease-specific differential DNA methylation in different neurodegenerative diseases. It is unlikely that the identified DNA methylation changes in *ANK1* could be useful as a biomarker clinically, as we have previously shown that *ANK1* is not hypermethylated in blood in AD(Lunnon et al., 2014). However, these epigenetic changes could represent novel therapeutic targets for disease, if shown to be causal in pathology. At present we are unable to determine whether these changes represent a cause or a consequence of the disease process. Further functional studies should therefore be performed to determine the potential disease causality of this modification.

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