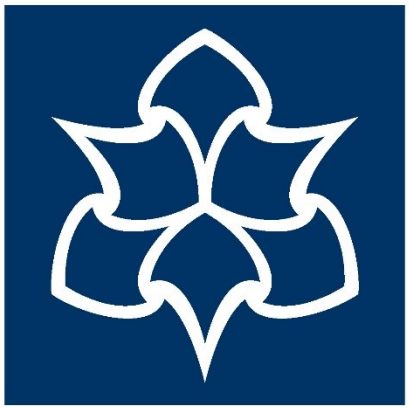
The regulation of brain-derived neurotrophic factor in cognitive ageing

Toby Aarons



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Abstract

Introduction: Brain-derived neurotrophic factor (BDNF) is essential for neurogenesis and synaptic plasticity, which are important in memory, particularly in neurons in the prefrontal cortex and hippocampus. Declining BDNF levels have been suggested to contribute to cognitive ageing, which in the context of a globally ageing population, needs to be investigated further, particularly in regards to underlying epigenetic regulation.

Methods: We investigated BDNF and cognitive ageing in the MyoAge population, which consisted of cognitively healthy young (n=135, 23.4 ± 2.7 years) and older (n=226, 74.5 ± 3.4 years) adults. Cognitive function had previously been tested using CANTAB. Investigation of BDNF and Alzheimer's disease was performed in the Dyne-Steele cohort (n=67). Bisulfite pyrosequencing was used to quantify *BDNF* methylation. Genotyping was performed on the *BDNF* Val66Met polymorphism. RT-PCR was used to quantify *BDNF* gene expression. Chromatin Immunoprecipitation and subsequent allele discrimination via pyrosequencing was used to determine gene-environment interactions.

Results: In the MyoAge cohort, we observed that *BDNF* exon I methylation is associated with cognitive decline in the male population, with higher *BDNF* exon I methylation associated with worse executive functioning and working memory capacity in the old males (P=0.001, P=0.011, respectively). Physical activity was associated with reduced methylation of *BDNF* exon IV in the older females (P=0.033). Early life education is associated with reduced *BDNF* methylation in the older adults. In the Dyne-Steele cohort, *BDNF* exon I expression was associated with faster rates of memory decline in the AD group. *BDNF* exon I methylation was associated with *BDNF* gene expression in the healthy group, but not the AD group. Histone H3K27ac marker bound more readily to the *BDNF* rs56133711 A allele.

Discussion: These results suggest that *BDNF* methylation is associated with cognitive decline in the older population. Lifestyle factors play a significant role in *BDNF* methylation and should be considered with further investigation of *BDNF* methylation and cognitive ageing. BDNF could act to compensate AD damage, however AD neuropathology appears to interfere with epigenetic regulation of *BDNF.*

1. Introduction

1.1 Ageing

In the past half century, ageing has emerged as one of the key policy issues of governments and organisations across the globe. This was highlighted by the production of international legal and policy framework by the United Nations General Assembly (United Nations, 2002) and The World Health Organisation (WHO, 2012), separately. The WHO (WHO, 2012) noted the challenges ahead, including the increased risk of disability with age and the increased need for provision of care to ageing populations, while proposing policy to combat this. Their health specific policy called for the prevention and reduction of the burden of excess disabilities, chronic disease and premature mortality, as well as a reduction of risk factors associated with major diseases while increasing factors that protect health throughout life.

However, the most recent World report on ageing and health (WHO, 2015), emphasised that there is still not enough being done to ensure that these policies are being implemented to a satisfactory level. Moreover, with the increased global situation of ageing (Figure 1.1), the challenge to combat the issues associated with an ageing population is becoming increasingly more complex.

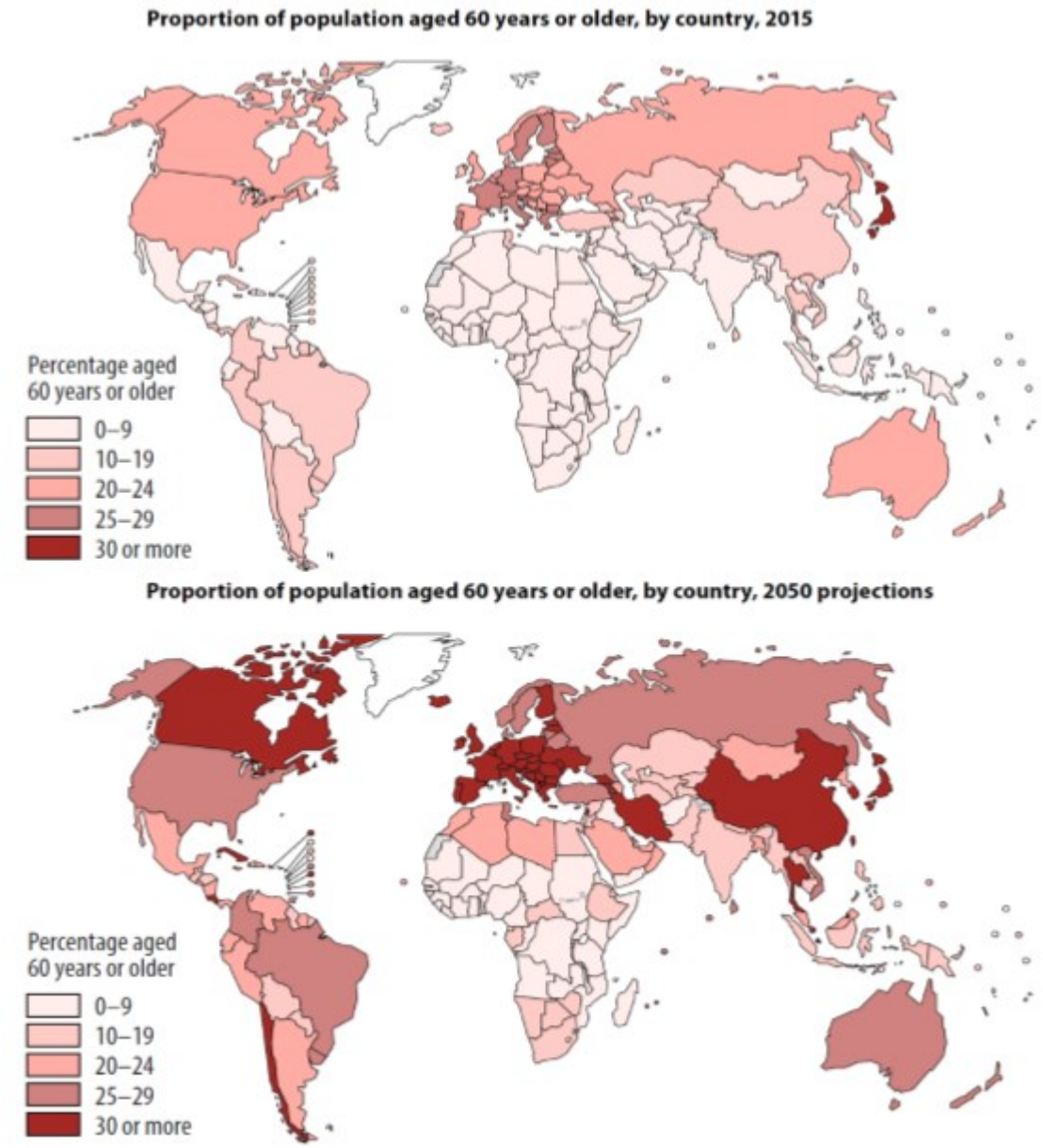

Figure 1.1. The proportion of the global population aged 60 years or older in 2015, based on data from individual countries, in comparison to 2050 projections. Figure was adapted from the 'World report on ageing and health' (WHO, 2015).

1.2 Age-related cognitive decline

Ageing is the lifelong accumulation of molecular and cellular damage, which eventually overcomes the mechanisms and safeguards working within the body to repair damage and remove waste products. The increasing imbalance gradually impairs and damages the systems required for healthy function, resulting in dysfunction, disease and ultimately death (Kirkwood, 2008). Cognitive function is one such system that is notoriously linked to ageing. It has been difficult to accurately determine the rate at which cognitive decline

will affect populations, mainly due to the inconsistent definitions that are supposed to separate normal brain ageing from the various stages of dementia onset (Sachdev et al, 2015). Moreover, the multifactorial nature of the phenomenon, including the impact of lifestyle factors for example, will need to be considered.

Cognitive ageing puts the elderly at risk of various impairments to quality of life, including social isolation and abuse (WHO, 2015). However, potentially the most detrimental risk of ageing and its associated cellular mechanisms is neurodegeneration (Alzheimer's Association, 2017). Therefore, with the current predictions of an increasingly ageing population, there should be concern for the ensuing burden of neurodegenerative disease.

1.3 Dementia

Dementia is a syndrome that affects the ability for someone to live a normal life, it is characterised by loss of memory and other cognitive abilities that are essential for everyday functioning. Of all the brain abnormalities that are defined under dementia, Alzheimer's disease (AD) is the most prevalent (Alzheimer's Association, 2017). AD is association of the accumulation of β-amyloid plaques and neurofibrillary (tau) tangles, which have been implicated in the interference of neuronal signalling and death.

The neuropathology of AD has been suggested to begin 20 years before clinical observations (Villemagne et al, 2013). This emphasises the potential cross-over with cognitive ageing, and with no successful treatments to reverse or alter the progression of AD, full understanding of this link in countering the growing global burden is essential. This should include the development of an advanced biomarker framework (Dubois et al, 2014).

1.4 Mechanisms involved in age-related cognitive decline

It is still uncertain to what extent current knowledge covers the processes involved in age-related cognitive decline, there are clearly missing pieces to the puzzle, but conversely significant discoveries have been and are being included into mechanistic pathways. Structurally, various reductions in synaptic density, including in the hippocampus and prefrontal cortex, important in memory and executive function, manifest with age

(Petralia et al, 2014), as well as atrophy within the above areas (Apostolova et al, 2012) and across the brain (Erten-Lyons et al, 2013). Adult neurogenesis, important for the formation of new memories such as in fear conditioning and spatial learning, decreases in an age-related manner (Seib and Martin-Villalba, 2015).

While cognitive ageing and AD are distinct conditions, the similarities are notable, from the atrophy or damage to the neurons associated in memory, such as in the prefrontal cortex. Therefore there may be underlying mechanisms that contribute to the progression of these disorders in similar ways.

1.5 BDNF

Brain-derived neurotrophic factor (BDNF) is a highly potent neurotrophin that promotes neurogenesis, synaptic plasticity and long-term potentiation (LTP) in the CNS (Barde et al, 1982; Kang and Schuman, 1995; Figurov et al, 1996). It binds with high affinity to the transmembrane tropomyosin-related kinase receptor (TrkB) on neurons and glia (Yan et al, 1997) which initiates downstream signalling cascades, including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MEK), and phospholipase C-γ (PLC-γ) associated pathways (Figure 1.2). Together, they promote further transcription, synaptic plasticity, protein-synthesis dependent plasticity, cell survival and neurogenesis (Yoshii and Constantine-Paton, 2010).

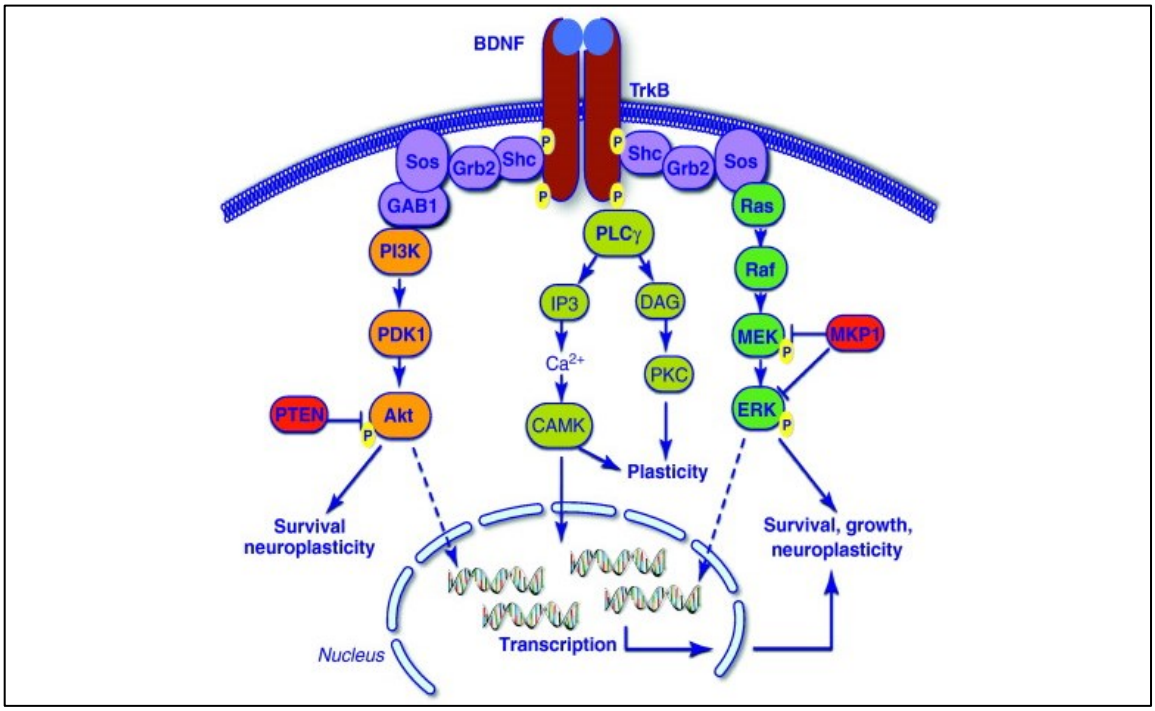

Figure 1.2 BDNF-TRKB downstream signaling cascade. Taken from Duman and Voleti (2012).

1.6 *BDNF* gene

The BDNF gene (Figure 1.3), on chromosome 11p13 (Maisonpierre et al, 1991), contains at least 9 functional promoters and 11 exons (Pruunsild et al, 2007). *BDNF* exon IX is the 3' coding exon which is alternatively spliced to one of the other 5' exons upstream, each of which respond to different cellular stimuli, allowing different transcripts to be generated that are stable in multiple intracellular environments (Pruunsild et al, 2007). It is this transcriptional responsiveness which allows BDNF to be secreted and function across multiple intra- and extra-cellular domains, primarily in neurons but it is also expressed peripherally, such as within endothelial cells and leukocytes (Gass and Hellweg, 2010).

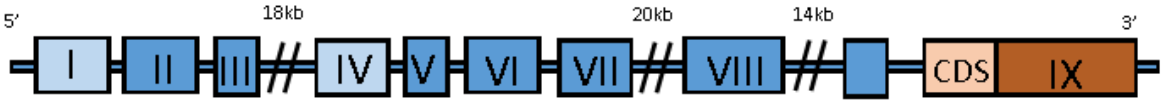

Figure 1.3. The BDNF gene . The structure of the BDNF gene, including the eight 5' exons and the 3' exon IX. Exons I and IV are highlighted in this study. Adapted from UCSC Genome Browser (https://genome.ucsc.edu/), the gene spans approximately 67,000 bp (chr11: 27,654,714-27,721,800, GRCh38/hg38)

1.7 BDNF is associated with cognitive ageing

BDNF has been implicated in the 'age-by-disease hypothesis', in which *BDNF* expression is reduced in the ageing brain and a reduction in *BDNF* expression has been associated with multiple neurological disorders (McKinney et al, 2015). BDNF is widely regarded for its influence on synaptic plasticity, which is essential for cognitive development and memory formation (Lu et al, 2014). BDNF is secreted from peripheral cells as well as neurons, which brings into question the role of peripheral BDNF in cognition. Notably, BDNF actively crosses the blood-brain barrier (BBB) in both directions (Poduslo et al, 1996; Pan et al, 1998), meaning peripheral BDNF is from the same reserve as BDNF within the CNS. Therefore the consensus is that peripheral BDNF directly influences neuronal and cognitive function.

The genetics of BDNF has also been considered to contribute to the different progression of cognitive ageing between individuals. The *BDNF* Val66Met polymorphism is located within the coding strand of the human BDNF gene (Figure 1.5) and is responsible for an amino-acid substitution of valine to methionine at codon 66. It has been observed to inhibit the activity dependent secretion of BDNF (Chen et al, 2004), and has been implicated in cognitive ageing (Miyajima et al, 2008; Lim et al, 2013).

Reductions in BDNF have been widely investigated as a mediator of age-associated decline in synaptic density and cognitive function (Gloriosso et al, 2011), with a significant association between BDNF and cognitive ageing being observed (Komulainen et al, 2008; Erickson et al, 2010; Oh et al, 2016). However, the underlying mechanisms behind age-associated BDNF declines were less understood, and so epigenetics was postulated as a potential regulator.

1.8 Epigenetics

Epigenetics, first defined by Conrad Waddington (1942) as "epi" or above "genetics" to bridge the disparity between genotype and phenotype, has emerged from controversial origins to become a field that could potentially progress the understanding of a vast amount of molecular and cellular processes.

Epigenetics can be defined as 'molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA sequence' (Skinner et al, 2010).

DNA methylation is by far the most studied epigenetic mechanism, in which DNA methyltransferases (DNMTs) transfer a methyl ($-CH_3$) group to the fifth carbon on the six-atom ring of cytosine, modifying it to 5-methylcytosine (5mC). This process only occurs to a cytosine in a CpG (5'-cytosine-phoshphate-guanine-3') dinucleotide. 5mC is abundant in the genome, consisting of ~1% of the total bases, which means almost 80% of human CpG dinucleotides are methylated (Ehrlich, 1982). However, potentially the areas of most interest are the predominantly unmethylated CpG islands (CGIs), which contain a high concentration of CpGs and are associated with the promoter regions of human genes (Bird, 2002; Illingworth et al, 2010; Maunakea et al, 2010). Methylated cytosines are associated with inhibition of transcription due to the function of the methyl group. They can prevent transcription factors from binding to DNA where they are present, and recruit methyl-binding proteins (MBPs), which also inhibit transcription factor binding as well as forming repressive protein complexes (Klose and Bird, 2006) (Cedar and Bergman, 2009).

Histone modifications encompass a vast array of changes that alter the structure of chromatin, some of which are displayed in Figure 1.4. One such modification is histone acetylation of the global histone domain and the N-terminal tails, which is regulated by histone acetyltransferases (HATs) and histone deacetylates (HDACs) (Bannister and Kouzarides, 2011). Modifications to lysine 27 of histone H3 (H3K27) have been found at *BDNF* promoters I, II, IV and VI in mature hippocampal neurons (Palomer et al, 2016). H3K27 tri- (me3) and di- (me2) methylation is associated with gene silencing, with H3K27me3 important in inhibiting transcription and cell stability (Farrari et al, 2013), whereas H3K27Ac is found in actively transcribed genes (Tie et al, 2009).

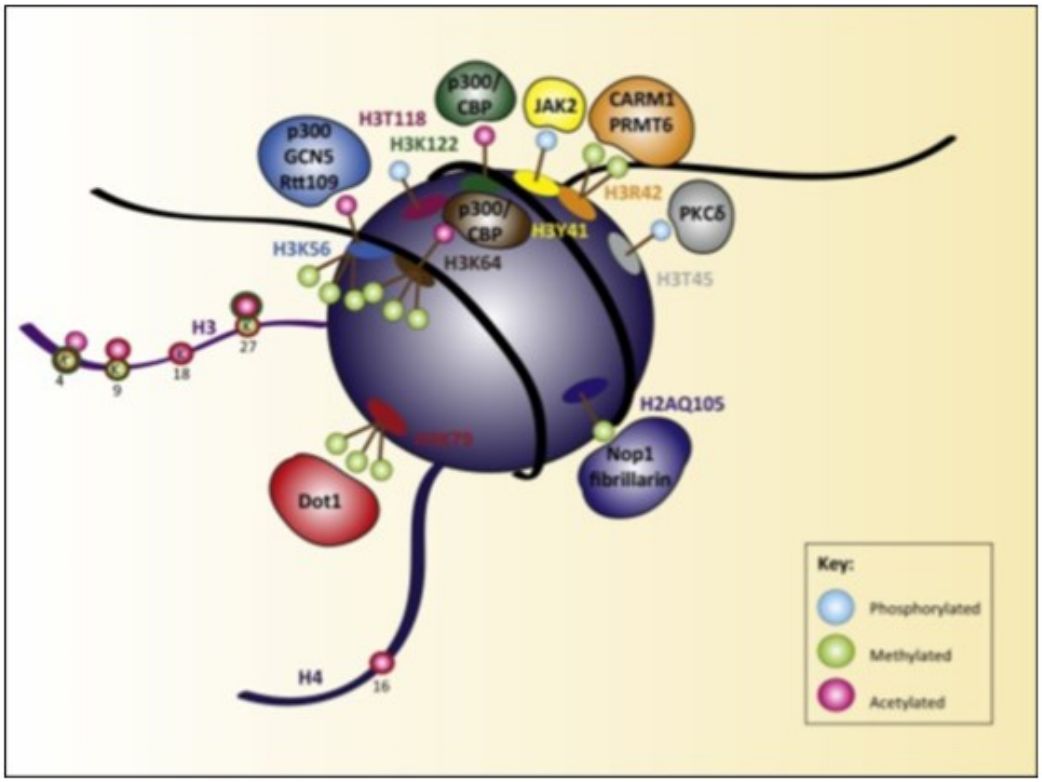

Figure 1.4. Schematic of global domain and histone tail modifications. The H3K27 tail modification corresponds with histone acetylation. Taken from Lawrence et al (2016).

1.9 *BDNF* epigenetics

Epigenetic mechanisms have been widely shown to regulate *BDNF* expression, with studies predominantly evaluating *BDNF* promoter methylation. With CGIs near many of the promoters of *BDNF*, including I and IV (Figure 1.5), methylation of the promoters is clearly important in regulating the different transcripts generated from their multiple cellular sources. *BDNF* methylation has been highly implicated in numerous neurological conditions, including depression, bipolar disorder and schizophrenia (Karpova et al, 2014; Zheleznyakova et al, 2016). Furthermore, *BDNF* methylation at exons I and IV (Figure 1.5) have been associated with learning and memory, and as *BDNF* methylation is dynamic, there is growing interest in investigating *BDNF* methylation in cognitive ageing, mediated by the age-associated decline in *BDNF* expression (Komulainen et al, 2008; McKinney et al, 2015).

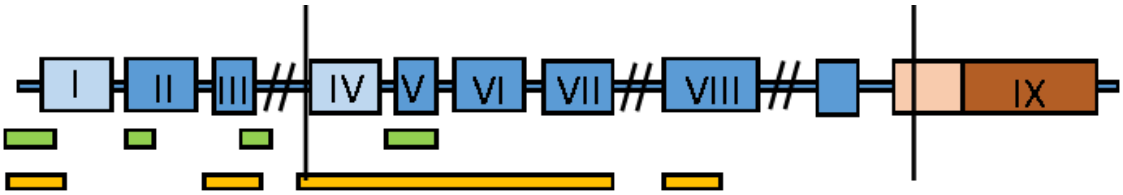

Figure 1.5. The BDNF gene regulated . The structure of the BDNF gene, including the eight 5' exons and the 3' exon IX. Exons I and IV are highlighted in this study. Green bars indicate CpG islands. Yellow bars indicate regions of high H3K27Ac binding. Pro-BDNF consists of both brown coding regions, which is cleaved to form mature BDNF, indicated by the dark brown box. The line prior to exon IV indicates rs56133711, while the line in the coding region indicates rs6265 (Val66Met). Adapted from UCSC Genome Browser (https://genome.ucsc.edu/).

DNA methylation of *BDNF* in the peripheral cells has been investigated as a biomarker for cognitive ageing, with good reason. It has been recently suggested that, even though *BDNF* methylation differs between neurons and peripheral cells (Davies et al, 2012), changes in promoter methylation of *BDNF* in the periphery could mirror changes in the brain (Davies et al, 2012; Kundakovic et al, 2015). Furthermore, with BDNF actively crossing the BBB, the epigenetic regulation of peripheral BDNF could affect the levels of BDNF within the brain. Notably, Chang et al (2014) have previously reported that higher *BDNF* promoter I methylation might be a risk factor for AD.

1.10 Overview and Aims

The regulation of the *BDNF* gene presents great promise as a biomarker for neuronal and cognitive ageing, with the dynamic *BDNF* methylation been implicated in ageing and risks of AD. However, there is limited research on the direct influence of *BDNF* regulation and the implication of cognitive ageing, as well as what role *BDNF* methylation actually plays in the onset of AD.

Therefore, the main aims of this thesis are:

- To develop the understanding of the role of BDNF in cognitive ageing
- To investigate how the epigenetic and genetic regulation of *BDNF* contributes to cognitive ageing
- To determine whether lifestyle factors, such as exercise, influenced the impact of short-term and long-term epigenetic changes on cognitive ageing
- To analyse the changes in *BDNF* methylation in relation to AD
- To determine whether genetic variations within the *BDNF* gene can influence its epigenetic regulation, and whether these interactions are contributing the cognitive ageing process

2. Methodology

2.1 Study Populations

2.1.1 MyoAge

Recruitment

The current study utilised samples from the previously performed MyoAge study including young (18-30 years) and old (69-81 years) adults, who were required to be physically and mentally healthy, who were recruited across Europe (Manchester, UK; Paris, France; Leiden, The Netherlands; Tartu, Estonia and Jyväskylä, Finland). Recruitment was standardised between all sites involved, using telephone-screening interviews and further screening the research centres, the full criteria were described by McPhee et al (2013). Characteristics are presented in Table 2.1, sourced from McPhee et al (2013). Physically active old adults were defined as having currently maintained moderate to vigorous activity for at least 3 years, with at least three 30 minute sessions per week, with the intent to improve health and fitness. Inactive old adults were defined as having only performed at maximum one session per week to improve health and fitness, for the past 3 months, while rarely being active in their daily lives (McPhee et al, 2013).

Ethical approval was granted from each institution involved in the cohort, and all participants provided written informed consent and were medically screened before participation.

Table 2.1. MyoAge cohort participant characteristics (n=361)

	Young (n=135)	Old (n=226)
Age (years), Mean (SD)	23.4 (2.74)	74.5 (3.38)
Females, n (%)	71 (52.6)	116 (51.3)
MyoAge Institution, n (%)		
Holland	33 (24.4)	71 (31.4)
Finland	34 (25.2)	68 (30.1)
Estonia	33 (24.4)	47 (20.8)
France	17 (12.6)	17 (7.5)
United Kingdom	18 (13.3)	23 (10.2)
Education[1], n (%)		
Basic School	0 (0)	33 (17.8)
High School	14 (12.3)	70 (37.8)
University	100 (87.7)	82 (44.3)
Mental State		
MMSE Score[2], median (IQR)	30 (29-30)	29 (28-30)
GDS Score[3], median (IQR)	0 (0-1)	1 (0-2)
Activity in old adults		
Active, n (%)	-	124 (54.9)
Inactive, n (%)	-	102 (45.1)
Methylation (%), mean (SD)		
BDNF exon I [4]	4.64 (1.32)	5.79 (2.17)
BDNF exon IV [5]	4.65 (1.85)	4.32 (1.32)
Val66Met genotype[6] Methionine allele present (AA/AG), n (%)	33 (24.4)	76 (33.6)

[1]Recorded data in young: n=114, and old: n=185. [2]Recorded data in young: n=134, and old: n=223.
[3]Recorded data in young: n=116, and old: n=200. [4]Recorded data in young: n=62, and the old: n=86.
[5]Recorded data in young: n=85, and the old: n=85. [6]Recorded data in young: n=127, and old: n=204.

Mental state and cognitive testing

The Geriatric Depression Scale (Yesavage et al, 1982) was used to screen for depression, with those with a score of 5 points or over excluded from the study. The Mini Mental State Examination (Folstein et al, 1975) was used to screen for cognitive impairment, where a score of 23 points or lower is attributable to factors other than healthy cognitive decline, and would exclude the participant from the study. The Cambridge Neuropsychological Test Automated Battery (CANTAB) device (Cambridge Cognition Ltd., Cambridge, UK) was used to measure various cognitive domains (Sahakian and Owen, 1992). Participants were given the following tests:

Spatial Span (SSP) is a test of working memory capacity (Owen et al, 1990). Briefly, participants were required to observe a series of white squares on the screen in a pseudo-random pattern. Some squares would briefly change colour in a randomly generated sequence, then a tone would signal the participant to attempt to repeat the pattern. A score was given based on the longest sequence successfully recalled by the participant.

One Touch Stockings of Cambridge (OTS) is a test of executive functioning, otherwise known as the planning task (Owen et al, 1995). The participants were presented with two displays on a screen (Figure 2.1) and they had to work out the minimum number of moves required to match the top display to the bottom. A score was given based on the number of tasks the participant correctly worked out on their first attempt.

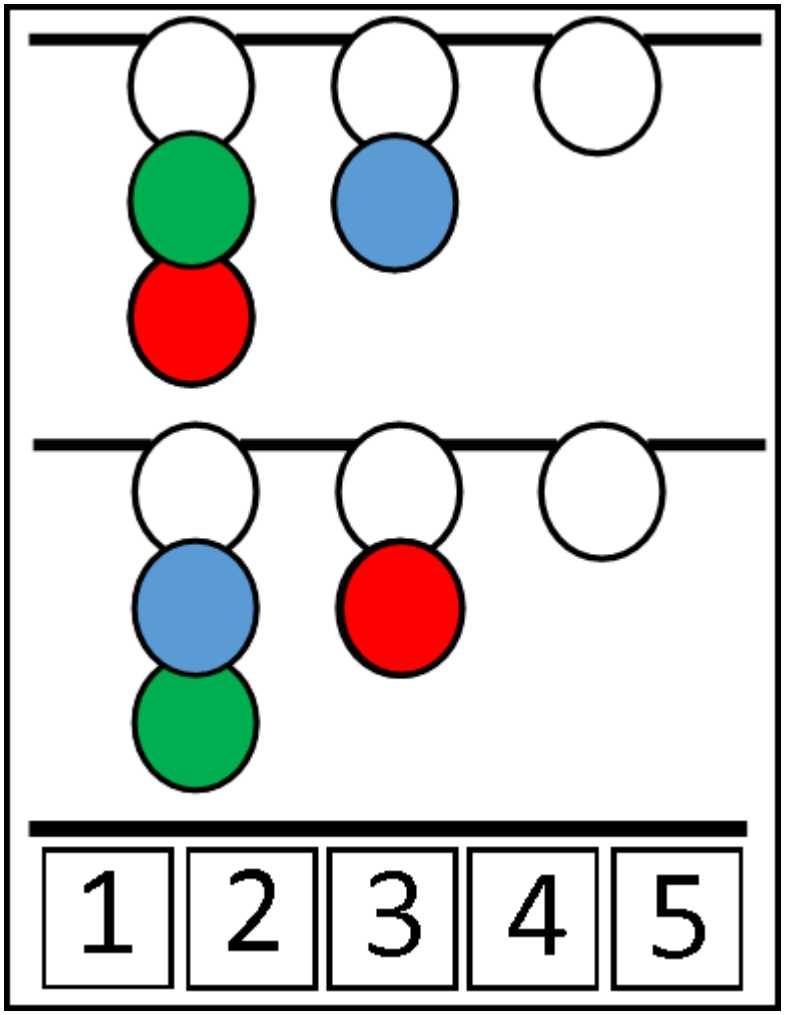

Figure 2.1. One Touch Stockings of Cambridge task. Participants had to work out in their heads the minimum number of moves required to match the top display to the bottom.

Paired Associates Learning (PAL) is a test of episodic memory (Owen et al, 1993). Participants were required to remember the location of specific patterns, which were contained within white boxes displayed on the screen. A score is worked out based on the number of errors accumulated, when incorrectly guessing which box contained a specific pattern.

2.1.2 Dyne-Steele

This study utilised samples of the prefrontal cortex donated by participants who had undergone a program of longitudinal testing as part of the Dyne-Steele cohort (Rabbitt et al, 2004). As part of this program, participants aged from 42 to 92 years, had undergone longitudinal cognitive testing across a period of 36 months to 20 years. Participants repeatedly filled out self-rating inventories throughout the study, including Yesavage depression (Yesavage et al, 1982), personal details and self-awareness questionnaires. From the cohort, 67 participants were analysed in this study. Their characteristics are displayed in table 2.2, sourced from Payton et al (2015). AD classification was determined based on the neuropathology scores following the National Institute on Aging-Alzheimer's Association guidelines (Hyman et al, 2012). Ethical approval was granted by the Manchester Brain Bank.

Table 2.2. Dyne-Steele cohort participant characteristics (n=67)

	Control (n=17)	AD (n=27)	Total (n=67)
Age at death (years), Mean (SD)	87.5 (5.35)	87.6 (6.94)	87.5 (6.07)
Females, n (%)	12 (70.6)	21 (77.8)	46 (68.7)
Neuropathology group, Mean (SD)			
Thal	0.88 (1.05)	3.63 (1.01)	2.16 (1.71)
Cerad	0.47 (0.51)	2.41 (0.50)	1.36 (1.07)
Braak	0.82 (0.39)	2.26 (0.45)	1.56 (0.79)
Methylation (%)(a), Mean (SD)			
BDNF exon I	-	-	3.48 (1.18)
BDNF exon IV	-	-	2.28 (0.25)
Brain weight (g)(b), Mean (SD)	-	-	1207.4 (137.4)
Cognitive decline (g-factor), Mean (SD)			
Fluid Intelligence	0.496 (0.734)	0.219 (0.699)	0.398 (0.757)
Vocabulary	0.647 (0.787)	0.488 (0.903)	0.531 (0.776
Speed	0.648 (0.693)	-0.051 (0.842)	0.186 (0.826)
Memory	0.429 (0.726)	0.536 (1.148)	0.459 (0.936)
rs56133711 genotype			
Varient allele present (AA/AG), n (%)	-	-	31 (46.3)

A) Recorded data: n=66. B) Recorded data: n=43

Cognitive scores for the participants were constructed in a previous study (Payton et al, 2015). Vocabulary was tested using the Mill Hill and Wais vocabulary tests (Raven, 1965; Rabbitt et al, 2004). Tests for fluid intelligence comprised the Alice Heim test 4 (Heim, 1970) and the Culture Fair Test (Cattell, 1949). Tests for cognitive speed comprised the Alphabet Coding Task (Savage, 1984) and the Random Letters test (Rabbitt et al, 2004). Memory scores were created based on tests involving Immediate Verbal Free Recall, Delayed Recall, spatial memory, semantic memory and prepositions (Rabbitt et al, 2004).

2.2 Blood extraction

Whole blood was extracted following an overnight fast and processed, as described in McPhee et al (2013). Aliquots of plasma and buff coat samples were requested for this current study and stored at -80°C for until further testing.

2.3 DNA extraction

Genomic DNA was extracted from the MyoAge blood samples prior to this study. Genomic DNA was extracted from brain samples with the Isolate II Genomic DNA Kit (Bioline, UK), according to the manufacturer's instructions. Briefly, samples were incubated with 25 µL of proteinase K enzyme and 200 µL of supplied lysis buffer (Buffer G3) at 70°C for 30 mins. Following sample lysis, the lysate was applied onto a spin column and centrifuged to allow DNA binding. Subsequent column washes were applied to remove unwanted proteins and salts. Finally, the columns were briefly dried and the DNA was eluted with 50 µL of pre-warmed elution buffer.

DNA quantification and purity was performed on the NanoDrop 2000c (Thermo Scientific, Wilmington, USA). The 260/280 and 260/230 ratios were used to indicate whether the samples were protein and salt free. Stock solutions were then aliquoted and stored at -20°C until further analysis.

2.4 Bisulfite treatment

Genomic DNA samples (10 µL) were bisulfite-converted using the EpiMark® Bisulfite Conversion Kit (New England Biolabs® Inc.) according to the protocol. DNA samples were combined with the prepared bisulfite mix and incubated in a thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA), with the specified cycling conditions, to deaminate the unmethylated cytosines to uracil (Frommer et al, 1992). Once converted, the bisulfite DNA was briefly mixed with DNA Binding Buffer before being transferred to the EpiMark spin columns for desulphonation and purification. A final volume of 40 µL was stored at -20°C.

2.5 Primer optomisation

BDNF promoter primers were designed via the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany). *BDNF* primers are presented in table 2.3 and sequences analysed are in Figure 2.2.

Table 2.3. Human *BDNF primers* used for bisulfite pyrosequencing

Primer	Sequence (5'-3')
BDNF exon I Forward	TGAGTGATGATTAAATGGGGATTG
BDNF exon I Forward	ACTATTAACTCACATTTAAAAAACCATAAC
BDNF exon I Forward	TGGGGATTGGGGGGA
BDNF exon IV Forward	GATTTTGGTAATTCGTGTATTAGAGTGTT
BDNF exon IV Forward	AGATTAAATGGAGTTTTCGTTGAT
BDNF exon IV Forward	AATGGAGTTTTCGTTGATGGGGTGCA

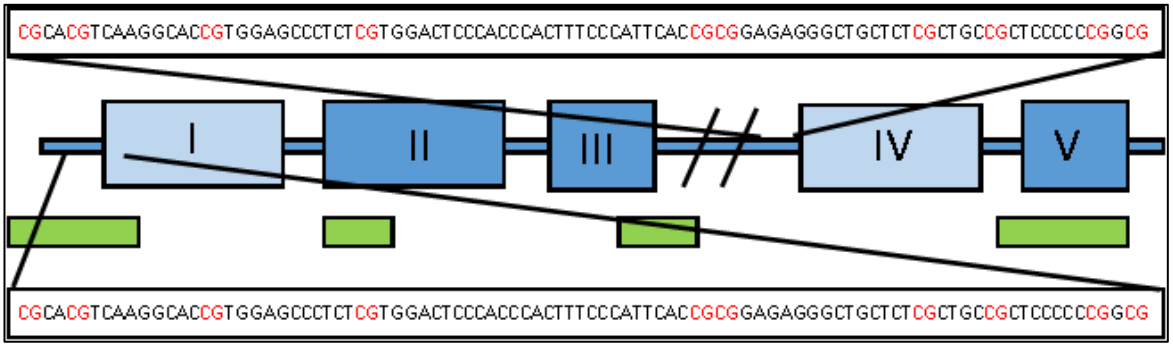

Figure 2.2. BDNF sequences analysed in bisulfite pyrosequencing. Sequences are in the promoter regions of *BDNF* exon I (Bottom) and *BDNF* exon IV (Top). Green- CGIs.

2.6 Polymerase chain reaction (PCR)

The PCR solutions were prepared as follows, *BDNF Ex1*: 2 µL bisulfite DNA, 10 µL MyTaq® HS mix (Bioline), 1 µL forward primer, 1 µL reverse primer and 6 µL nuclease-free H_2O; *BDNF Ex4:* 2 µL bisulfite DNA, 10 µL MyTaq® HS mix (Bioline), 0.5 µL forward primer, 0.5 µL reverse primer and 7 µL nuclease-free H_2O. Total reaction volumes were 20 µL. Solutions were prepared on ice and spun down into 96-well plates prior to amplification in the thermocycler (Table 2.4; SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA). PCR products were stored at -20°C.

Table 2.4. PCR cycling conditions for *BDNF* exon I and IV

Step	Description	Temperature (°C)	Time	Number of cycles per step
1	Initial Denaturation	95	15 minutes	1 cycle
2*	Denaturation Annealing Extension	94 56 72	30 seconds 30 seconds 45 seconds*	50 cycles*
3	Final Extension	72	10 minutes	1 cycle

Conditions above for *BDNF* exon I. **BDNF* exon IV was similar apart from 49 cycles and 30 seconds extension at Step 2

2.7 Agarose gel electrophoresis

Gels contained 2% agarose and Midori Green Advance DNA Stain (NIPPON Genetics Europe) within 1x tris-borate-EDTA (TBE) buffer. PCR products were mixed with 5x DNA loading buffer (Bioline) and were loaded against a 50 bp DNA hyperladder (Bioline). Gels were imaged using the Chemedoc® Touch imaging system (Bio-Rad).

2.8 Bisulfite pyrosequencing

For epigenetic analysis, DNA methylation and allele discrimination within the *BDNF* promoters was determined using bisulphite pyrosequencing, on the Pyromark Q24 system (Qiagen, Hilden, Germany). The process, involving the use of PyroMark Q24 Advanced CpG reagents, was performed in accordance with the manufacturer's protocol. Reagents included a sequencing master mix prepared by mixing Streptavidin Sepharose High Performance beads (GE Healthcare Biosciences), Pyromark binding buffer and nuclease-free H_2O in a ratio of 1:40:24 µL, respectively. This master mix was combined with 15 µL of PCR product in a 24-well PCR plate, which was then shaken at 1400 RPM for 10 minutes on the TS-100 thermos-shaker (BioSan) at room temperature. Immediately after agitation, the samples were processed using the PyroMark Q24 workstation. The vacuum was applied to the PCR plate for 15 seconds to ensure all the solution had been aspirated and that all the beads had been captured on the filter probes. The filter probes were subsequently flush with 70% ethanol, denaturation solution and wash buffer for 5, 5 and 10 seconds respectively. The filter probes were drained and the beads were deposited by shaking the probes in the PyroMark Q24 Plate, containing the diluted sequencing primer (0.3 µM). The PyroMark Q24 Plate was heated at 80°C for 2 minutes (DB-2D, Dri-Block®, Techne) and then allowed to cool to room temperature. The PyroMark Q24 Advanced reagents and nucleotides were loaded into the PyroMark Q24 Cartridge according the PyroMark Q24 Advanced software (assay design). The PyroMark Q24 Advanced software (analysis mode) was used to quantify methylation levels and grade the quality of analysis at each variable site.

2.9 Genotyping

Genotyping of the MyoAge cohort was performed on the functional polymorphism, Val66Met (dbSNP: rs6265), using a commercial genotyping assay (KASP™, LGC)). Genotyping was performed in accordance with the manufacturer's protocol. Rs6265 genotyping mix was prepared with DNA (5 ng/µL), 2x KASP Master Mix, KASP Assay (primer) mix and water at a ratio of 2, 5, 0.14 and 3 µL respectively. Total reaction volumes were 10 µL. Solutions were prepared on ice and spun down into 96-well plates prior to amplification in the Stratagene Mx3000P qPCR system (Agilent). The PCR cycle conditions are displayed in table 2.5.

Table 2.5. PCR cycling conditions for KASP genotyping

Step	Description	Temperature (°C)	Time	Number of cycles per step
1	Hot-start activation	94	15 minutes	1 cycle
2	Denaturation Annealing / Elongation	94 61-55	20 seconds 60 seconds (drop 0.6°C per cycle)	10 cycles
3#	Denaturation Annealing / Elongation	94 55	20 seconds 60 seconds	26 cycles
4#*	Denaturation Annealing / Elongation	94 57	20 seconds 60 seconds	3 cycles

Based on LGC manual. # After these steps, the plate was cooled to 30°C for 1 minute, in order to read the plate *Step 4 was only required as a recovery if clear genotyping clusters were not obtained, it could be repeated until clear clusters were obtained.

2.10 RNA extraction

Brain tissue (30 mg) was cut on dry ice and stored in sterile 1.5 mL tubes at -80°C. RNA extraction was performed in accordance with the manufacturer's protocol, based on the method by Chomczynski and Sacchi, 1987. Prior to extraction, tissue was stored in dry ice to minimise RNA degradation, and immediately homogenised in 1 mL TRIsure™ (Bioline, UK), pipetting the solution to break down the tissue. After 5 minutes of incubation at room temperature, 0.2 mL of chloroform (Thermo Fisher) was added to the solution, which was covered and shaken vigorously for 15 seconds, followed by a further 3-minute incubation at room temperature, allowing the water to separate from the chloroform. The solution was centrifuged at 12,000 g for 15 minutes at 4°C, separating the proteins and lipids, DNA and RNA. The RNA was carefully transferred, without disturbing the

interphase, to a sterile 1.5 mL tube and mixed with 0.5 mL cold isopropyl alcohol. The samples were incubated for 10 minutes at room temperature, to allow precipitation to occur, and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was carefully removed, 1 mL of 75% ethanol was added to wash the remaining pellet by vortex and the tubes were centrifuged at 8,000 g for 5 minutes at 4°C. The ethanol wash was repeated once more, after which the ethanol was removed by pipetting and air-drying until just the pellet remained. The pellet was dissolved in 20 µL of nuclease-free H_2O at -80°C. RNA concentrations and purity was quantified using the Nanodrop 2000c (Thermo Scientific, Wilmington, USA).

2.11 Reverse transcription

The Tetro cDNA synthesis kit (Bioline, UK) was used to reverse transcribe the extracted RNA samples, according to the manufacturer's protocol. In each well of a sterile 96-well PCR plate, 2 µg total RNA was mixed by pipetting with 1 µL random hexamers, 1 µL 10mM dNTP mix, 4 µL 5x RT buffer, 1 µL RiboSafe RNase Inhibitor, 1 µL Tetro reverse transcriptase (200 u/µL) and 10 µL DEPC-treated water. Total reaction volume was 20 µL. Solutions were spun down and incubated in the thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) at 25°C for 10 minutes, 45°C for 30 minutes and 85°C for 5 minutes. Samples were diluted with 80 µL nuclease-free H_2O and stored at -20°C.

2.12 Real-time polymerase reaction (RT-PCR)

Relative gene expression was analysed with qPCR using SensiFAST™ SYBR® Lo-ROX kit (Bioline), in accordance with the manufacturer's protocol. The cDNA primers were designed using the NCBI primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with the criteria based of the MIQE guidelines (Bustin et al, 2009), including primers spanning at least one exon junction and a 'GC clamp' at the 3' end of the primers. Primers are presented in table 2.6.

Table 2.6. Human *BDNF transcript primers* used for RT-PCR

Primer	Sequence (5'-3')	Primer efficiencies (%)
BDNF transcript I Forward	CAGCATCTGTTGGGGAGACGA	94.7
BDNF transcript I Reverse	GCCACCTTGTCCTCGGATGT	
Accession number	NM_170731.4	
Product size	180bp	
BDNF transcript IV Forward	TGGGAGTTTTGGGGCCGAAG	101.7
BDNF transcript IV Reverse	TGGTCATCACTCTTCTCACCTGG	
Accession number	NM_170733.3	
Product size	102bp	
BDNF total Forward	GGACCCTTCAGAGGTGGCTC	104.4
BDNF total Reverse	GTCGGCTTGAGTGTGGTCCT	
Product size	123bp	
ACTB Forward	CATCCTCACCCTGAAGTACC	92.0
ACTB Reverse	ATAGCAACGTACATGGCTGG	
Accession number	NM_001101.3	
Product size	219bp	
GAPDH Forward	CCGCATCTTCTTTTGCGTCG	91.0
GAPDH Reverse	TGGAATTTGCCATGGGTGGA	
Accession number	NM_002046.5	
Product size	102bp	

The PCR solutions were prepared as follows: 2 µL cDNA template, 10 µL SensiFAST™ SYBR® Lo-ROX Mix (Bioline), 0.5 µL forward primer, 0.5 µL reverse primer and 7 µL nuclease-free H_2O; *BDNF Ex4:* 2 µL bisulfite DNA, 10 µL MyTaq® HS mix (Bioline), 0.5 µL forward primer, 0.5 µL reverse primer and 7 µL nuclease-free H_2O. Total reaction volumes were 20 µL. Solutions were prepared on ice and spun down into 96-well plates prior to amplification in the Stratagene Mx3000P qPCR system (Agilent). The PCR cycle conditions are displayed in table 2.7.

Table 2.7. PCR cycling conditions for RT-PCR

Step	Description	Temperature (°C)	Time	Number of cycles per step
1	Polymerase activation	95	2 minutes	1 cycle
2	Denaturation Annealing Extension	95 60* 72	5 seconds 10 seconds 10 seconds	40 cycles
3	Melt-profile analysis	95 55 95	1 minute 30 seconds 1 minute	1 cycle

**BDNF* exon I PCR was performed with an annealing temperature of 62°C

Every plate included every analysed sample in duplicate, as well as 2-4 calibration samples and 2-4 non-template control samples. The $2^{-\Delta\Delta Ct}$ equation was used to determine relative RNA gene expression (Livak and Schmittgen, 2001), normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB).

2.13 Chromatin Immunoprecipitation (ChIP)

Brain tissue (30 mg) was cut on dry ice and stored in sterile 1.5 mL tubes at -80°C. ChIP was performed according to the methodology of Murgatroyd et al (2012), with revisions listed below.

The chromatin was sheared using the M220 Focused-ultrasonicator (Covaris), to target a DNA fragment range of 300-1000 bp with the following settings: water bath temperature- 6°C, processing time- 8 minutes, duty cycle- 5%, peak incident power- 75 watts, and cycles per burst- 200. The remaining debris was pelleted by centrifuging at 10,000 g for 10 minutes at 4°C, and the supernatant with the sheared protein-DNA complex was transferred to new tubes and stored at -80°C.

For immunoprecipitation, 5 µL supernatant was stored for an input control. Rabbit IgG antibody (1 µL) was used for the negative control, while the anti-Histone H3K27ac antibody (Abcam, ab4729; 5 µL) was used for the pull-down samples.

Immune complexes were mixed at 65°C for 30 minutes, and then the beads were captured on the magnetic rack, where the supernatant was transferred to a new tube. At this point, the input control was diluted with 95 µL elution buffer (to 100 µL). To reverse the formaldehyde cross-linking, NaCl (5M, 6 µL) was added to all the tubes and the complexes were incubated at 65°C for 4 hours. This was followed by the addition of Proteinase K (2.5 µL, 20 mg/ml, Bioline) and incubation at 65°C for 2 hours to degrade any remaining bound protein.

2.14 Statistical analysis

The IBM SPSS Statistics software (v.22) was used to analyse all associations. Normal distribution of data was determined using the Sharipo-Wilk test for normality. Significance was determined by $P<0.05$.

Differences in plasma BDNF between different levels of educational attainment were determined using Kruskal-Wallis H tests. All other differences in plasma BDNF levels between categorical variables were determined using Mann-Whitney U tests. Relationships with plasma BDNF and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age as described in the results.

Cognitive function Z-scores were created against the average of the young populations, stratified by gender. Differences in cognitive function between different levels of educational attainment were determined using Kruskal-Wallis H tests. Differences in cognitive function between the Val66Met genotype while controlling for age were determined using an ANCOVA. All other differences in cognitive function between categorical variables were determined using Mann-Whitney U tests. Relationships with cognitive function and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age as described in the results.

Differences in *BDNF* methylation levels between different levels of educational attainment were determined using Kruskal-Wallis H tests. All other differences in *BDNF* methylation levels between categorical variables were determined using Mann-Whitney U tests. Relationships with *BDNF* methylation levels and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age as described in the results.

Differences in total *BDNF* expression between neuropathology scores and the rs56133711 genotype were determined using Kruskal-Wallis H tests, with Tukey HSD multiple comparisons performed for post-hoc analysis. All other differences in total *BDNF* expression between categorical variables were determined using Mann-Whitney U tests.

Relationships with total *BDNF* expression and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age and neuropathology as described in the results. Differences in *BDNF* exons I and IV expression between neuropathology scores and the rs56133711 genotype were determined using one-way ANOVAs, with multiple Mann-Whitney U tests with Bonferroni adjustments performed for post-hoc analysis. All other differences in *BDNF* exons I and IV expression between categorical variables were determined using independent samples t-tests. Relationships with *BDNF* exons I and IV expression and continuous variables were determined using Pearson correlations, with Pearson partial correlations used when controlling for age and neuropathology as described in the results.

Relationships with cross-sectional and longitudinal vocabulary score and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age and neuropathology as described in the results. Relationships between all other Dyne-Steele cognitive scores and continuous variables were determined using Pearson correlations, with Pearson partial correlations used when controlling for age and neuropathology as described in the results.

Relationships with all individual CpG methylation levels and ranked variables were determined using Spearman correlations, with Spearman partial correlations used when controlling for age and neuropathology as described in the results. Relationships with *BDNF* average methylation and continuous variables were determined using Pearson correlations, with Pearson partial correlations used when controlling for age and neuropathology as described in the results.

BDNF rs56133711 allelic discrimination was determined using a paired-samples T-test.

3. Results

3.1 Association of age and gender on BDNF plasma levels

A prior analysis of the sets of BDNF values attained from across the different MyoAge institutions, showed that the BDNF levels from the Estonian institute were almost 5-fold higher than the other institutions (figure 3.1, P<0.0001). Therefore, further analyses within this study omitted the Estonian measurements.

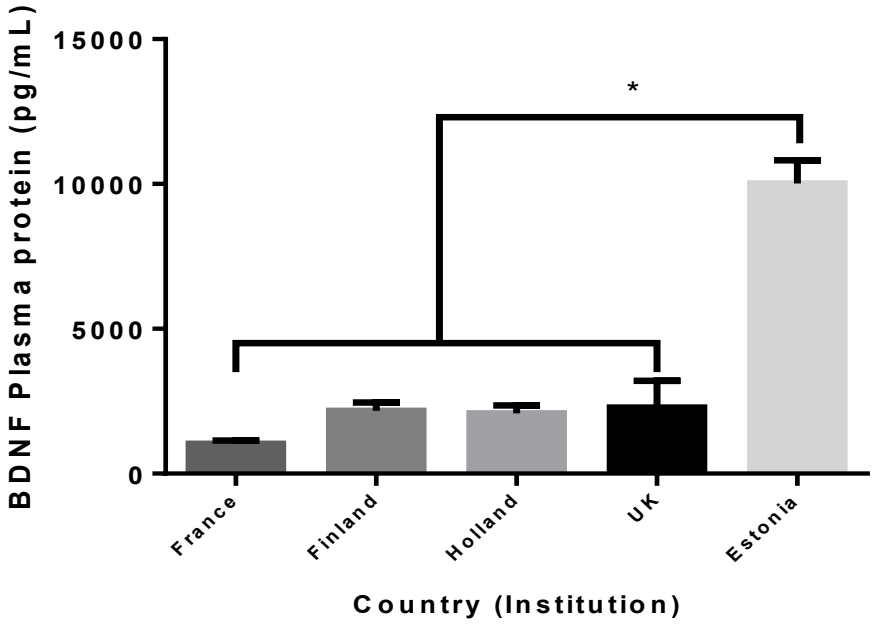

Figure 3.1 Analysis of BDNF plasma protein taken from the MyoAge institutions. BDNF protein levels taken from the Estonian institute were almost three standard deviations from the mean of the other MyoAge institutions, *P<0.0001

BDNF protein levels, previously measured in the plasma extracts, were tested between the young and the old populations within the MyoAge cohort. Notably, BDNF levels were significantly higher in the older population (P=0.003). When the population was stratified by gender (figure 3.2), the significant increase in BDNF levels was attributed to the male population (young: 1010.99 pg/mL, old: 2417.72 pg/mL, P<0.001), while no significant change was found within the female population (young: 1946.58 pg/mL, old: 2216.77, P=0.347). BDNF levels were also significantly higher in the young females than in the young males (male: 1010.99 pg/mL, female: 1946.58 pg/mL, P=0.034).

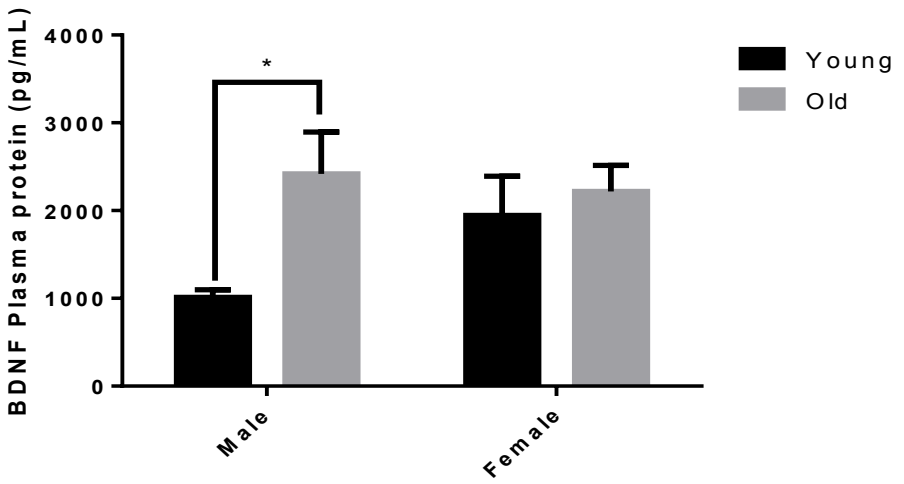

Figure 3.2. Analysis of BDNF plasma protein between the young and old adults, stratified by gender. Data is presented as mean and standard deviation. * P<0.001.

3.2 The relationship between plasma BDNF and cognitive function

The relationship between cognitive performance and age within the MyoAge cohort has been described previously (Bradburn et al, 2016), indicating that the older population performed worse on the tests of working memory, episodic memory, executive functioning and the combined global cognition.

BDNF levels from the males and females were analysed against the results from the cognitive tests (working memory, episodic memory, executive functioning and the combined global cognition) using Spearman partial correlation while controlling for age, the results are displayed in Table 3.1. No significant correlations were found with BDNF

Table 3.1 Relationship between BDNF plasma protein and cognitive functioning (stratified by gender)

	Male		Female	
	BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)	
	r	*P*	*r*	*P*
Working memory capacity	-0.052	0.560	-0.048	0.572
Executive functioning	-0.001	0.992	-0.119	0.156
Episodic memory	0.058	0.506	-0.109	0.194
Global cognition	0.022	0.804	-0.142	0.090
MMSE	0.033	0.706	0.132	0.117

r= Spearman correlation coefficient. P= significance

across the tests (Figure 3.3).

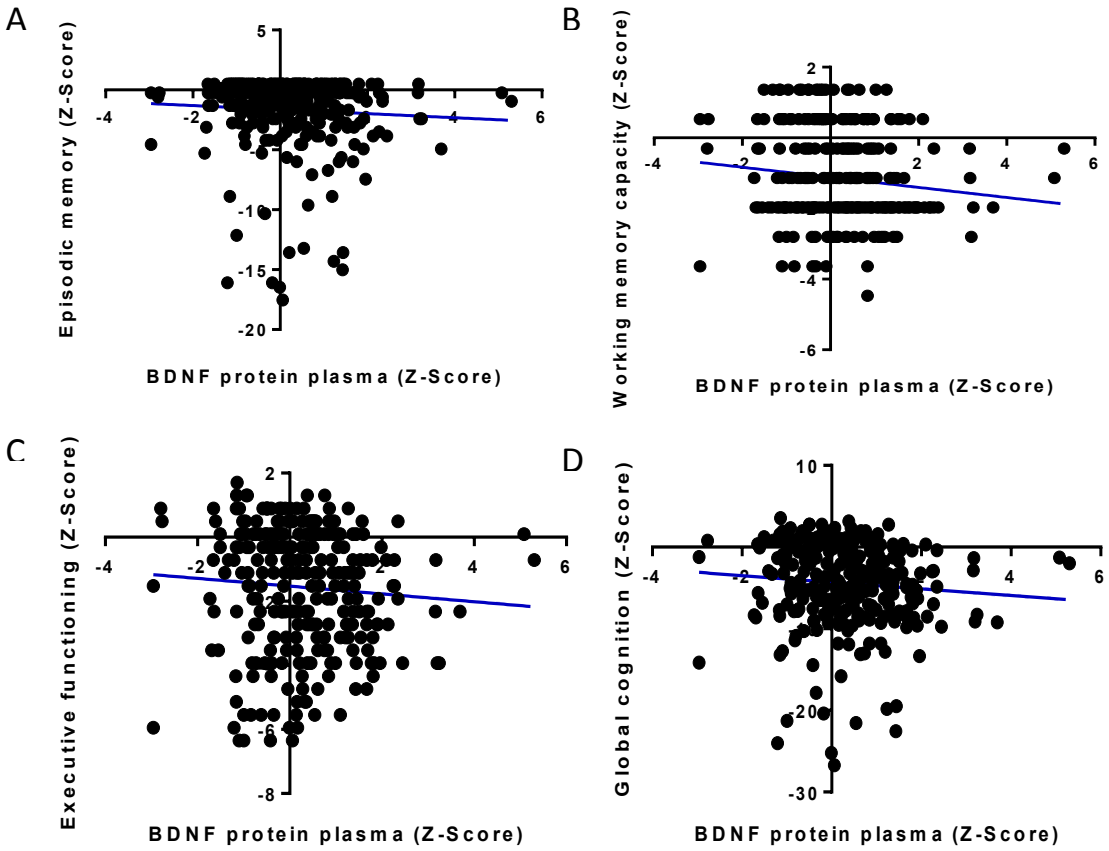

Figure 3.3. Relationship between BDNF plasma protein and cognitive function. A) Relationship between BDNF plasma protein and episodic memory. B) Relationship between BDNF plasma protein and working memory capacity. C) Relationship between BDNF plasma protein and executive functioning. D) Relationship between BDNF plasma protein and global cognition. No significant correlations were found.

When BDNF levels from the males and females of the older group were analysed against the cognitive tests, it was found that there was a significant positive association between MMSE scores and plasma BDNF levels in the old females (Table 3.2).

Table 3.2 Relationship between BDNF plasma protein and cognitive functioning (stratified by age and gender)

	Male		Female	
	BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)	
	r	*P*	*r*	*P*
Young				
Working memory capacity	-0.182	0.207	-0.247	0.077
Executive functioning	-0.149	0.301	-0.145	0.305
Episodic memory	0.062	0.667	-0.176	0.212
Global cognition	-0.151	0.294	-0.262	0.061
MMSE	-0.205	0.153	-0.062	0.663
Old				
Working memory capacity	0.097	0.374	0.003	0.977
Executive functioning	-0.010	0.930	0.081	0.445
Episodic memory	0.062	0.572	-0.067	0.528
Global cognition	0.093	0.393	-0.046	0.666
MMSE	0.083	0.446	0.234	**0.025**

r= Spearman correlation coefficient. P= significance

MMSE scores are predictive of cognitive function in the MyoAge cohort and importantly in the old females (Table 3.3). This suggests that levels of plasma BDNF may predict cognitive performance in older females.

Table 3.3 Relationship between MMSE scores and cognitive functioning in old females

	Old female adults	
	MMSE score	
	r	*P*
Working memory capacity	0.156	0.098
Executive functioning	0.166	0.080
Episodic memory	0.173	0.066
Global cognition	0.190	**0.044**

r= Spearman correlation coefficient. P= significance

3.3 Correlation between methylation at individual CPG sites within promoters I and IV of the *BDNF* gene in the blood

Following the finding that BDNF protein levels may correlate with cognition, we investigated the possibility that *BDNF* gene regulatory potential might differ. In this regard, we studied epigenetic marking of the *BDNF* gene, specifically DNA methylation. The *BDNF* gene has multiple promoters linked to different untranslated first exons. We focused our attention on the promoters linked to the alternative first exons I and IV (Figure 1.3, introduction).

Within the promoters of exons I and IV we analysed 5 and 9 CpG sites, respectively. In the region analysed within the *BDNF* exon I promoter, the methylation of all the CpG sites were significantly correlated with one another (Figure 3.4 left, r>0.490, p<0.001). This was also the case with the *BDNF* exon IV promoter region (Figure 3.4 right, r>0.290, p<0.001). Therefore, an average methylation score could be made for both regions.

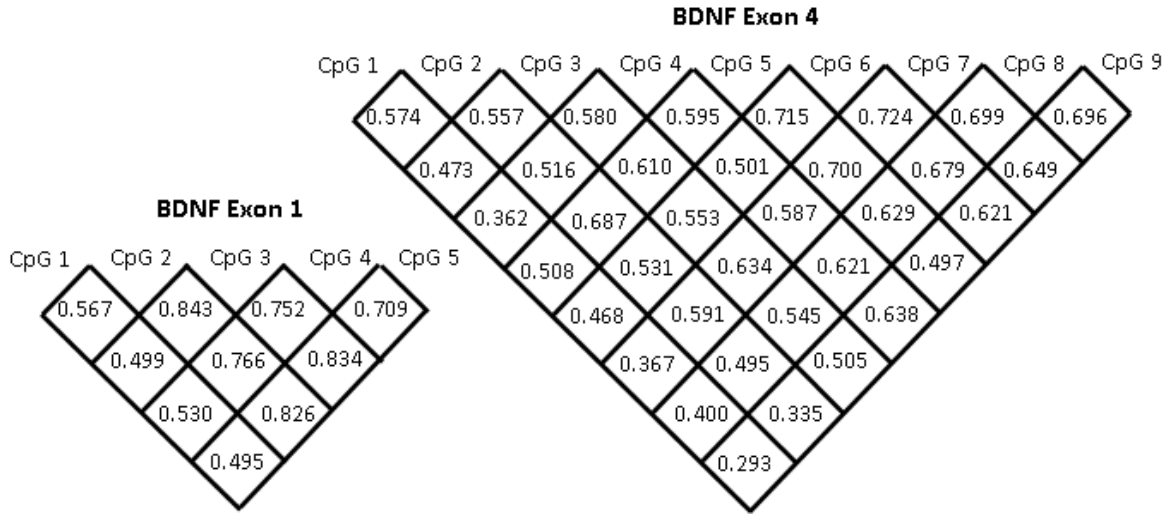

Figure 3.4. Spearman's correlation between CpGs in BDNF exon I (left) and IV (right). All correlations were significant (p<0.001)

3.4 Relationship between *BDNF* methylation in the blood and age

Methylation in the promotor region of BDNF exon I was significantly higher in the older population in four out of the five CpG sites and across the average of the location. No significant changes were seen in any of the CpG sites in the promoter region of exon IV (Table 3.4, Figure 3.5).

Table 3.4 Relationship between *BDNF* methylation and age

		Young adults		Old adults		
		n	Methylation (Z-Score)	n	Methylation (Z-Score)	*P*
BDNF Exon I						
	CpG 1	59	2.99 (0.00)	86	3.48 (0.20)	0.664
	CpG 2	59	5.27 (0.00)	86	6.46 (0.58)	**0.011**
	CpG 3	59	5.72 (0.00)	86	7.29 (0.71)	**<0.001**
	CpG 4	59	4.1 (0.00)	86	5.23 (0.79)	**0.001**
	CpG 5	59	5.11 (0.00)	86	6.47 (0.65)	**0.001**
	Average	59	4.64 (0.00)	86	5.79 (0.96)	**<0.001**
BDNF Exon IV						
	CpG 1	68	4.99 (0.00)	74	4.85 (-0.04)	0.910
	CpG 2	68	3.11 (0.00)	74	2.76 (-0.06)	0.603
	CpG 3	68	3.84 (0.00)	74	3.41 (-0.14)	0.235
	CpG 4	68	6.53 (0.00)	74	5.67 (-0.30)	0.076
	CpG 5	68	3.79 (0.00)	74	3.22 (-0.21)	0.228
	CpG 6	68	2.66 (0.00)	74	2.88 (-0.02)	0.804
	CpG 7	68	6.29 (0.00)	74	5.54 (-0.20)	0.115
	CpG 8	68	4.05 (0.00)	74	3.78 (-0.24)	0.079
	CpG 9	68	6.64 (0.00)	74	6.80 (-0.10)	0.515
	Average	68	4.65 (0.00)	74	4.32 (-0.19)	0.324

BDNF methylation is presented as a percentage, with Z-Scores based of the average of the young male and female populations separately. P values were determined by independent samples Mann-Whitney U tests

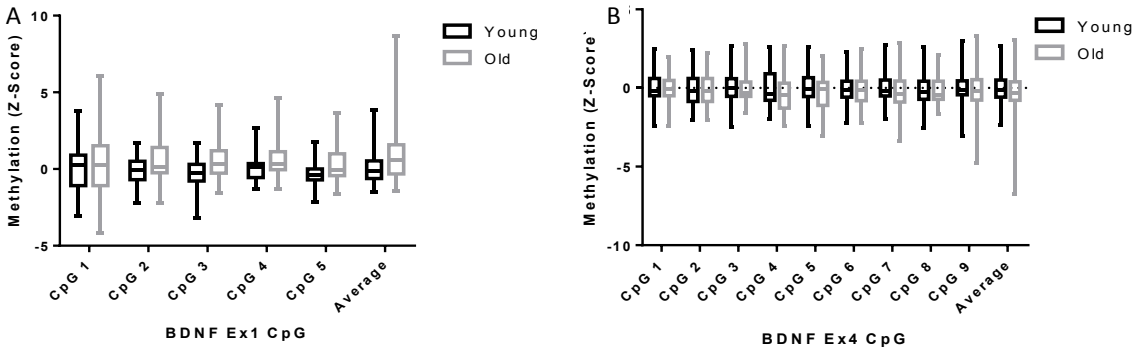

Figure 3.5. Relationship between BDNF methylation and age. A) Relationship between *BDNF* exon I methylation and age, old adults had significantly higher *BDNF* exon I methylation than the young adults (Average: P<0.001) B) Relationship between *BDNF* exon IV methylation and age, no significant differences were observed.

The differences in *BDNF* methylation in exon I between age groups were then analysed within the male and female populations, separately (Table 3.5, Figure 3.6). Notably, the vast majority of the significant increase in *BDNF* methylation in the older population was attributed to the female population. Within the female population, four out of the five CpG sites had significantly higher methylation levels. In the male population, methylation had also increased with age, with a significant increase at CpG 3 and smaller trends across the other sites.

Table 3.5 Relationship between *BDNF* exon I methylation and age, stratified by gender

		Male			Female		
		Methylation (Z-Score)		P value	Methylation (Z-Score)		P value
		Young	Old		Young	Old	
BDNF Exon I							
	CpG 1	3.15 (0.00)	3.42 (0.09)	0.909	2.87 (0.00)	3.53 (0.29)	0.527
	CpG 2	5.48 (0.00)	6.08 (0.18)	0.381	5.10 (0.00)	6.78 (0.90)	**0.006**
	CpG 3	5.89 (0.00)	7.01 (0.53)	**0.031**	5.59 (0.00)	7.52 (0.87)	**0.001**
	CpG 4	4.24 (0.00)	5.15 (0.50)	0.073	4.00 (0.00)	5.30 (1.03)	**0.002**
	CpG 5	5.52 (0.00)	5.97 (0.24)	0.304	4.80 (0.00)	6.87 (0.98)	**0.001**
	Average	4.86 (0.00)	5.53 (0.44)	0.068	4.47 (0.00)	6.00 (1.39)	**0.001**

BDNF methylation is presented as a percentage, with Z-Scores based of the average of the young male and female populations separately. P values were determined by independent samples Mann-Whitney U tests

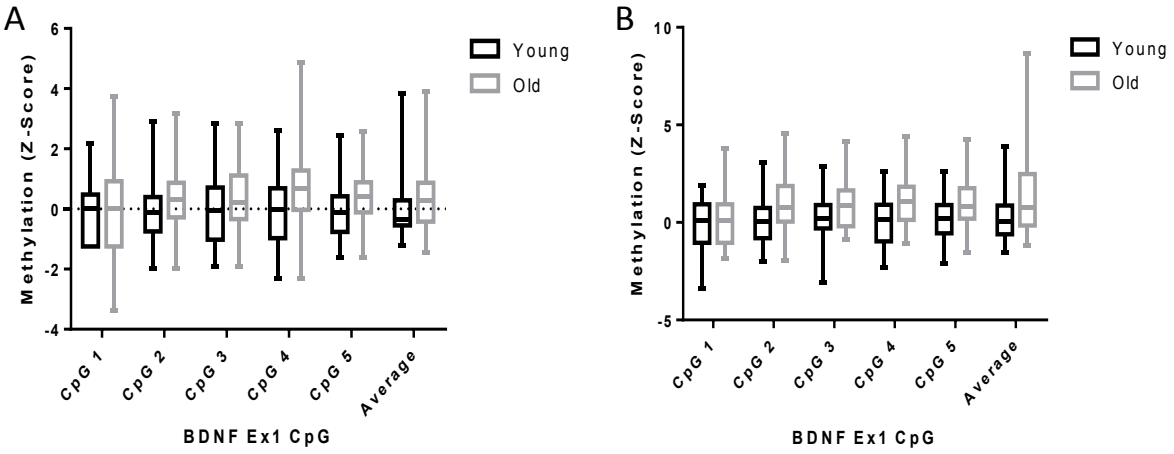

Figure 3.6. Relationship between BDNF exon I methylation and age, in males and females.
A) Young and old males, old males had marginally higher *BDNF* exon I methylation than the young males (Average: P=0.068) B) Young and old females, old females had significantly higher *BDNF* exon I methylation than the young females (Average: P=0.001).

3.5 Relationship between *BDNF* methylation and BDNF protein levels in the plasma

To determine whether *BDNF* methylation was influencing BDNF protein levels within the plasma, Spearman's correlations were performed between the methylation scores of *BDNF* exon I and IV, and BDNF protein levels, while controlling for age. No significant relationships were found between *BDNF* methylation and BDNF protein levels (Table 3.6, Figure 3.7).

Table 3.6 Relationship between *BDNF* methylation and BDNF plasma protein, stratified by gender

		Male		Female	
		BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)	
		r	*P*	*r*	*P*
BDNF Exon I					
	CpG 1	-0.030	0.826	-0.163	0.195
	CpG 2	0.021	0.881	-0.108	0.388
	CpG 3	0.064	0.636	-0.136	0.277
	CpG 4	-0.086	0.524	-0.088	0.480
	CpG 5	0.058	0.671	-0.026	0.838
	Average	-0.018	0.892	-0.091	0.466
BDNF Exon IV					
	CpG 1	0.043	0.744	0.068	0.557
	CpG 2	0.062	0.642	-0.114	0.348
	CpG 3	-0.087	0.514	-0.016	0.890
	CpG 4	0.022	0.868	0.053	0.647
	CpG 5	0.092	0.494	-0.006	0.957
	CpG 6	0.033	0.819	0.042	0.729
	CpG 7	0.011	0.934	0.091	0.436
	CpG 8	0.015	0.910	0.143	0.225
	CpG 9	-0.039	0.771	0.140	0.233
	Average	-0.032	0.807	0.095	0.416

r= Spearman correlation coefficient

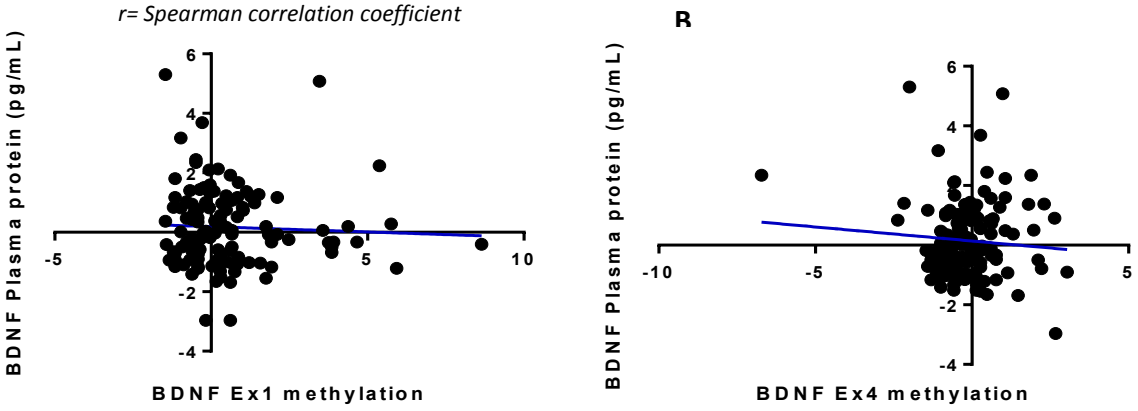

Figure 3.7. Relationship between BDNF methylation and plasma BDNF Left) Relationship between *BDNF* exon I methylation and plasma BDNF. Right) Relationship between *BDNF* exon IV methylation and plasma BDNF. No significant associations were observed.

With the previous observation that plasma BDNF was positively associated with MMSE scores within the old females, the population was further stratified by age, in order to determine whether accumulated *BDNF* methylation had a specific effect on plasma BDNF in later life. Further analysis showed that there were no significant associations between *BDNF* methylation and plasma BDNF levels in the old male or old female populations (Table 3.7), meaning methylation at these specific regions was not contributing to the differences in plasma BDNF in the old females, that was associated with improved cognitive function.

Table 3.7 Relationship between *BDNF* methylation and BDNF plasma protein (stratified by age and gender)

		Young male		Old male		Young female		Old female	
		BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)		BDNF plasma level (Z-Score)	
		r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
BDNF Exon I									
	CpG 1	0.139	0.536	-0.131	0.453	-0.251	0.227	-0.122	0.454
	CpG 2	0.046	0.842	-0.037	0.833	-0.061	0.766	-0.073	0.655
	CpG 3	0.095	0.675	-0.057	0.744	-0.115	0.576	-0.093	0.566
	CpG 4	-0.034	0.880	-0.206	0.235	-0.106	0.605	-0.036	0.826
	CpG 5	-0.140	0.533	0.100	0.566	-0.071	0.732	0.064	0.697
	Average	0.078	0.730	-0.132	0.450	-0.038	0.855	-0.051	0.757
BDNF Exon IV									
	CpG 1	0.139	0.481	-0.188	0.295	0.017	0.919	0.110	0.504
	CpG 2	0.562	**0.002**	-0.441	**0.012**	-0.236	0.179	0.006	0.973
	CpG 3	-0.042	0.833	-0.147	0.431	-0.054	0.756	0.024	0.887
	CpG 4	0.225	0.249	-0.147	0.429	-0.089	0.600	0.132	0.425
	CpG 5	0.427	**0.026**	-0.251	0.174	0.013	0.939	-0.016	0.925
	CpG 6	0.103	0.640	-0.144	0.456	-0.006	0.975	0.044	0.798
	CpG 7	0.095	0.639	-0.041	0.812	0.001	0.997	0.112	0.496
	CpG 8	0.139	0.481	-0.074	0.687	0.097	0.579	0.150	0.361
	CpG 9	-0.095	0.636	-0.045	0.806	0.005	0.976	0.243	0.136
	Average	0.030	0.881	-0.172	0.338	0.012	0.945	0.164	0.318

r= Spearman correlation coefficient

3.6 Relationship between *BDNF* methylation in the blood and cognition

Though there was no significant relationship between *BDNF* methylation and peripheral BDNF protein levels, we investigated whether *BDNF* methylation may still function as a marker for cognitive differences. Spearman partial correlations were performed between *BDNF* exon I and IV methylation, and the tests of cognitive function, while controlling for age. Methylation of *BDNF* exon I was negatively associated with executive functioning scores, however no significant associations were found with *BDNF* exon IV (Table 3.8, Figure 3.8).

Table 3.8 Relationship between *BDNF* methylation and cognitive function

		Executive Functioning		Working memory capacity		Episodic Memory		Global Cognition	
		r	P	*r*	P	*r*	P	*r*	P
BDNF exon I									
	CpG 1	-0.089	0.290	0.024	0.771	-0.016	0.850	-0.018	0.829
	CpG 2	-0.232	**0.005**	-0.050	0.550	-0.042	0.620	-0.116	0.166
	CpG 3	-0.205	**0.014**	-0.098	0.244	-0.041	0.624	-0.133	0.112
	CpG 4	-0.216	**0.009**	-0.060	0.472	-0.098	0.243	-0.144	0.085
	CpG 5	-0.239	**0.004**	-0.108	0.197	-0.072	0.391	-0.178	**0.033**
	Average	-0.218	**0.009**	-0.075	0.370	-0.057	0.497	-0.133	0.112
BDNF exon IV									
	CpG 1	-0.088	0.301	-0.043	0.616	0.013	0.878	-0.079	0.352
	CpG 2	-0.089	0.294	-0.067	0.428	-0.026	0.764	-0.089	0.297
	CpG 3	0.014	0.865	0.009	0.915	0.037	0.664	0.037	0.661
	CpG 4	-0.015	0.858	0.023	0.789	0.016	0.847	-0.005	0.950
	CpG 5	-0.067	0.428	0.050	0.557	-0.072	0.395	-0.073	0.393
	CpG 6	-0.112	0.188	0.030	0.726	-0.042	0.625	-0.080	0.343
	CpG 7	-0.073	0.387	0.023	0.789	0.001	0.987	-0.058	0.493
	CpG 8	-0.116	0.170	-0.013	0.876	-0.022	0.792	-0.068	0.421
	CpG 9	-0.042	0.620	-0.047	0.580	-0.129	0.126	-0.091	0.284
	Average	-0.071	0.401	-0.005	0.957	-0.046	0.590	-0.081	0.339

r= Spearman's correlation coefficient

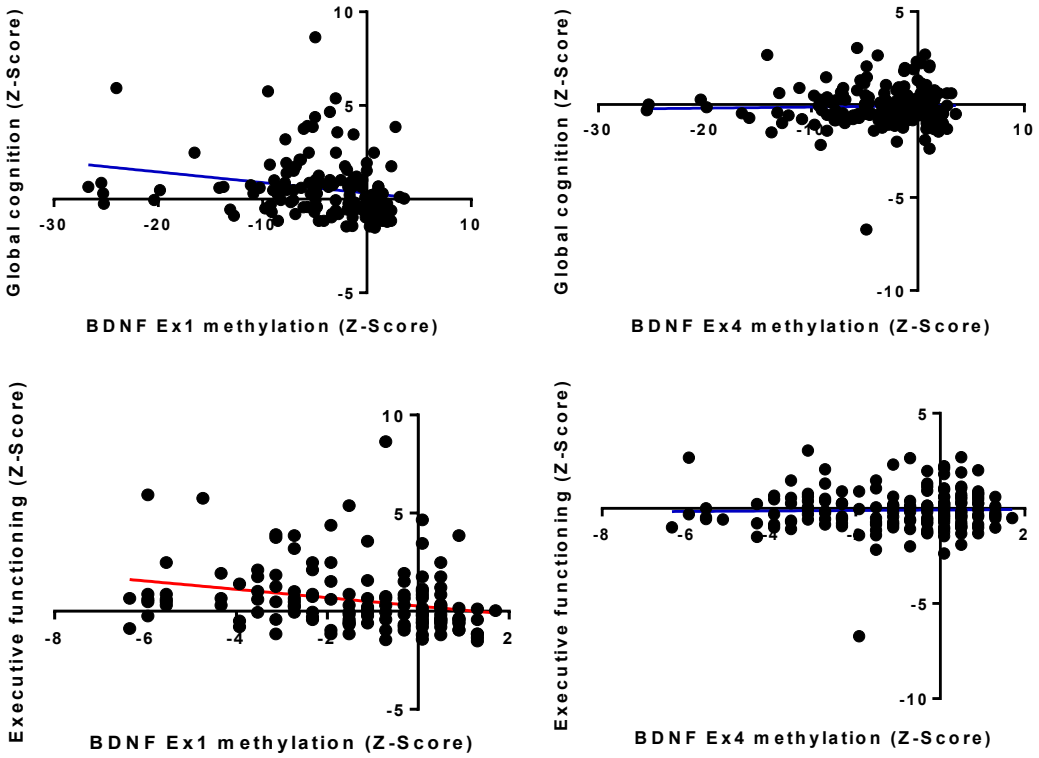

Figure 3.8. Relationship between BDNF methylation and cognitive function Top) Relationship between *BDNF* exons I and IV methylation and global cognition. Bottom) Relationship between *BDNF* exons I IV methylation and executive functioning. *BDNF* exon I was significantly associated with executive function (P=0.009)

When the population was stratified by gender, the negative association previously displayed between *BDNF* exon I methylation and executive functioning was attributed to the Male population, with further negative trends displayed between *BDNF* exon I and working memory capacity and global cognition scores (Table 3.9).

Table 3.9 Relationship between *BDNF* exon I methylation and cognition, stratified by gender

		Executive functioning		Working memory capacity		Episodic memory		Global cognition	
		r	P	*r*	P	*r*	P	*r*	P
Male									
	CpG 1	-0.210	0.096	-0.157	0.216	-0.197	0.119	-0.153	0.227
	CpG 2	-0.347	**0.005**	-0.134	0.291	-0.044	0.727	-0.163	0.199
	CpG 3	-0.470	**<.001**	-0.237	0.060	-0.065	0.608	-0.283	**0.023**
	CpG 4	-0.407	**0.001**	-0.216	0.086	-0.111	0.381	-0.219	0.082
	CpG 5	-0.363	**0.003**	-0.141	0.265	-0.029	0.819	-0.170	0.180
	Average	-0.467	**<.001**	-0.255	**0.042**	-0.083	0.516	-0.256	**0.041**
Female									
	CpG 1	0.035	0.759	0.240	**0.033**	0.075	0.513	0.126	0.269
	CpG 2	-0.159	0.163	0.072	0.530	-0.102	0.371	-0.108	0.341
	CpG 3	0.003	0.982	0.066	0.564	-0.096	0.402	-0.021	0.856
	CpG 4	-0.088	-0.088	0.089	0.437	-0.081	0.478	-0.055	0.631
	CpG 5	-0.154	0.175	0.001	0.997	-0.173	0.127	-0.163	0.150
	Average	-0.070	0.538	0.102	0.373	-0.083	0.469	-0.041	0.722

r= Spearman's correlaton coefficient

It was found that when the male population was stratified by age, there was still a strongly significant negative association between *BDNF* exon I methylation and executive functioning in the old males (Table 3.10), as well as a significant negative association between *BDNF* exon I methylation and working memory capacity. This suggests that *BDNF* exon I methylation could predict certain aspects of cognitive function in old male adults.

Table 3.10 Relationship between *BDNF* exon I methylation and cognition in males, stratified by age

		Male							
		Executive functioning		Working memory capacity		Episodic memory		Global cognition	
		r	P	*r*	P	*r*	P	*r*	P
Young									
	CpG 1	-0.312	0.113	-0.082	0.683	-0.322	0.102	-0.363	0.062
	CpG 2	-0.211	0.300	0.275	0.173	-0.159	0.439	0.065	0.754
	CpG 3	-0.259	0.192	0.252	0.205	-0.088	0.663	0.011	0.957
	CpG 4	-0.165	0.410	0.241	0.225	-0.356	0.069	0.017	0.933
	CpG 5	-0.153	0.445	0.173	0.390	0.008	0.970	0.038	0.849
	Average	-0.299	0.130	0.210	0.294	-0.245	0.217	-0.068	0.735
Old									
	CpG 1	-0.346	**0.031**	-0.282	0.082	-0.110	0.507	-0.193	0.238
	CpG 2	-0.415	**0.009**	-0.278	0.087	0.059	0.759	-0.170	0.300
	CpG 3	-0.570	**<0.001**	-0.438	**0.005**	-0.054	0.745	-0.333	**0.038**
	CpG 4	-0.446	**0.004**	-0.300	0.063	0.024	0.887	-0.171	0.297
	CpG 5	-0.464	**0.003**	-0.327	**0.042**	-0.005	0.977	-0.220	0.179
	Average	-0.524	**0.001**	-0.403	**0.011**	0.006	0.973	-0.237	0.147

r= Spearman correlation coefficient

3.7 Relationship between cognition and physical activity in the old adults

Cognitive function and ageing is not an independent process from the rest of the human biological systems, one of the many reasons peripheral BDNF is being analysed within this study. However, it also requires the consideration of other factors that could be impacting this cognitive ageing, either via independent pathways or through the mediation of BDNF.

When this study analysed the effect of exercise on cognitive function in the old adults, no significant differences were presented (Table 3.11).

Table 3.11 Relationship between cognitive function and activity in the older population

	Active		Inactive		
	n	Z-Score	n	Z-Score	*P*
Executive functioning	124	-2.709	102	-2.847	0.529
Working memory capacity	124	-1.897	102	-1.963	0.387
Episodic memory	124	-3.242	102	-3.648	0.713
Global cognition	124	-7.848	102	-8.458	0.353

Cognitive function tests are presented as Z-Scores, based on the average of the young male and female populations separately. P values were determined by independent samples Mann-Whitney U tests

3.8 Relationship between plasma BDNF and physical activity in the old adults

While the definitions of physically active and less active in the MyoAge population were well defined (McPhee et al, 2013), most of the participants self-assessed their physical activity levels, meaning some participants performing a similar amount of physical activity may have reported themselves differently. It is currently not known whether there is a minimum threshold for exercise to induce neurological changes, and so we do not know whether the two groups were significantly different to place either side of such a threshold. More likely it is a scaled change, therefore we still analysed BDNF against physical activity to see if the exercise was producing changes that could prelude an improvement in cognitive function. BDNF protein levels were investigated between the active and inactive populations of the old adults, although no significant change was observed (Figure 3.9, P=0.138).

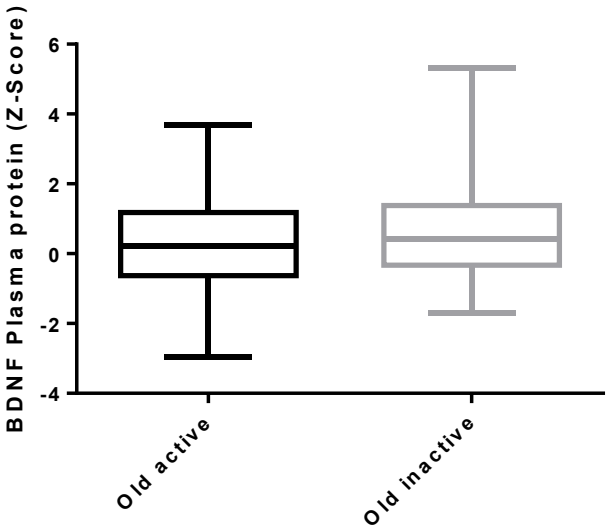

Figure 3.9. Relationship between Plasma BDNF and exercise. No significant difference was observed between the old active and old inactive populations.

3.9 Relationship between *BDNF* methylation in the blood and physical activity in the old adults

Though no significant difference was observed between BDNF protein levels and activity in the old adults, we next analysed the *BDNF* methylation levels between the old active and old inactive populations (Table 3.12). No significant differences were found across the *BDNF* exon I region. However, the old active population had reduced methylation

across the *BDNF* exon IV region, at CpGs 1, 2, 5, 6, 7, 8 as well as with the average (Figure 3.10, P<0.01).

Table 3.12 Relationship between *BDNF* methylation and activity in the old adults

		Active		Inactive		
		n	Methylation (Z-Score)	n	Methylation (Z-Score)	*P*
BDNF Exon I						
	CpG 1	44	3.33 (0.072)	42	3.64 (0.330)	0.315
	CpG 2	44	6.18 (0.410)	42	6.75 (0.748)	0.099
	CpG 3	44	7.16 (0.666)	42	7.43 (0.766)	0.467
	CpG 4	44	5.23 (0.752)	42	5.24 (0.823)	0.521
	CpG 5	44	6.26 (0.567)	42	6.68 (0.729)	0.305
	Average	44	5.63 (0.859)	42	5.95 (1.058)	0.346
BDNF Exon IV						
	CpG 1	42	4.33 (-0.352)	43	5.35 (0.269)	**0.005**
	CpG 2	40	2.45 (-0.256)	41	3.07 (0.095)	**0.013**
	CpG 3	42	3.38 (-0.224)	39	3.44 (0.061)	0.138
	CpG 4	42	5.50 (-0.512)	41	5.84 (0.068)	0.065
	CpG 5	41	2.86 (-0.557)	41	3.58 (0.179)	**0.001**
	CpG 6	40	2.71 (-0.208)	39	3.05 (0.202)	**0.033**
	CpG 7	42	5.10 (-0.425)	42	5.98 (0.079)	**0.012**
	CpG 8	42	3.52 (-0.498)	42	4.02 (0.064)	**0.024**
	CpG 9	42	6.71 (-0.169)	41	6.88 (0.044)	0.180
	Average	42	4.06 (-0.319)	43	4.58 (0.257)	**0.010**

BDNF methylation is presented as a percentage, with Z-Scores based of the average of the young male and female populations separately. P values were determined by independent samples Mann-Whitney U tests

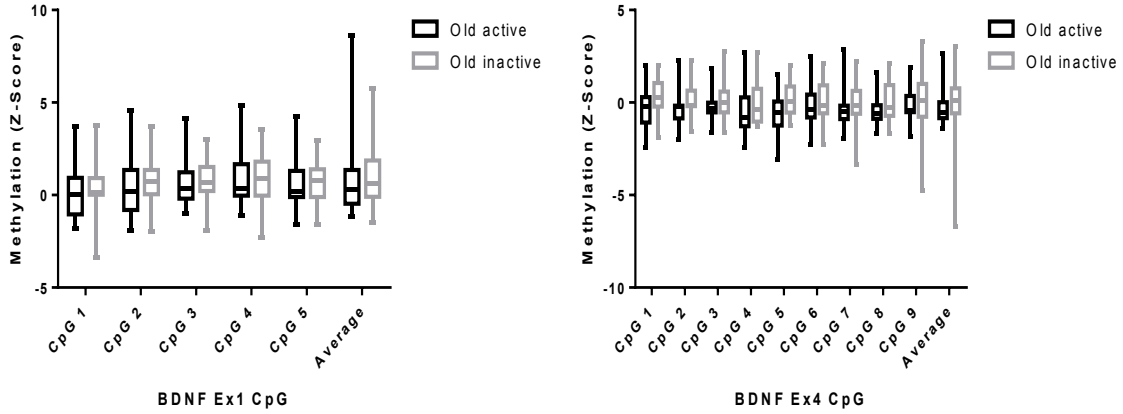

Figure 3.10. Relationship between BDNF methylation and activity. Left) Relationship between *BDNF* exon I methylation and activity, no significant differences were observed. Right) Relationship between *BDNF* exon IV methylation and activity, the old active group had significantly reduced *BDNF* exon IV methylation (Average: P<0.010).

When the population was further stratified by gender (Table 3.13), the difference between *BDNF* exon IV methylation and activity was maintained within the female population, with the average methylation of the sites significantly lower in the active population in comparison to the inactive (Figure 3.11). The same trend was still observed in the male population, however only one CpG site maintained a significant difference. Interestingly, the female population also displayed a reduction in *BDNF* exon I methylation, with a trend showing across the region (Average-P=0.073).

Table 3.13 Relationship between *BDNF* methylation and activity on the old adults, stratified by gender

		Male			Female		
		Active	Inactive	*P*	Active	Inactive	*P*
BDNF Exon I	n	16	23		28	19	
	CpG 1	0.159	0.040	0.779	0.022	0.681	0.067
	CpG 2	0.094	0.245	0.302	0.591	1.357	0.084
	CpG 3	0.509	0.548	0.806	0.756	1.030	0.260
	CpG 4	0.702	0.356	0.806	0.781	1.388	0.151
	CpG 5	0.228	0.256	0.942	0.761	1.301	0.052
	Average	0.466	0.414	0.841	1.083	1.837	0.073
BDNF Exon IV	n	14	19		25	16	
	CpG 1	-0.144	0.381	0.129	-0.468	0.136	0.056
	CpG 2	0.396	0.296	0.719	-0.621	-0.143	**0.009**
	CpG 3	0.103	0.180	0.972	-0.408	-0.080	0.057
	CpG 4	-0.279	0.404	0.245	-0.643	-0.331	0.249
	CpG 5	-3.410	0.475	**0.035**	-0.678	-0.172	**0.028**
	CpG 6	0.237	0.400	0.522	-0.458	-0.034	**0.043**
	CpG 7	-0.172	0.357	0.102	-0.566	-0.250	0.067
	CpG 8	-0.411	0.316	0.075	-0.547	-0.235	0.255
	CpG 9	0.030	0.260	0.545	-0.279	-0.212	0.596
	Average	0.099	0.591	0.216	-0.553	-0.140	**0.033**

BDNF methylation is presented as Z-Scores, based on the average of the young male and female populations separately.
P values were determined by independent samples Mann-Whitney U tests

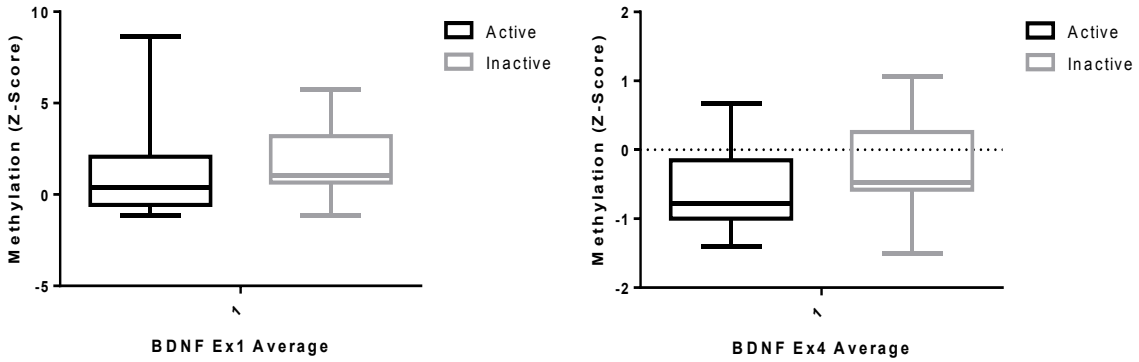

Figure 3.11. Relationship between BDNF methylation and activity, in the female population. Left) Relationship between *BDNF* exon I methylation and activity, in the female population, there was significantly higher methylation in the old inactive population (P=0.033). Right) Relationship between *BDNF* exon IV methylation and activity, in the female population, there was marginally higher methylation in the old inactive population (P=0.073).

3.10 Relationship between education and cognitive function, in the old adults

Education is an environmental factor that contributes to cognitive reserve, with further education producing long-term cognitive benefits and protection against neurodegeneration.

In the MyoAge cohort, old adults performed significantly better in all the tests of cognition, the further they had progressed their education (Table 3.14).

Table 3.14 Relationship between education and cognitive function, in the old adults

	Basic School		High School		University		
	n	Z-Score	n	Z-Score	n	Z-Score	*P*
Executive functioning	33	-3.055	70	-2.737	82	-2.173	**0.030**
Working memory capacity	33	-2.173	70	-1.947	82	-1.727	**0.014**
Episodic memory	33	-4.866	70	-2.241	82	-2.664	**0.003**
Global cognition	33	-10.094	70	-6.925	82	-6.502	**0.008**

P values were determined by independent samples Kruskal-Wallis H tests

Notably, this improvement in cognitive function with further education was only attributed to the female population (Table 3.15). No significant differences were observed within the old male population. However, there was a trend of better performance with University education against basic education in the males (Mann Whitney U: Executive functioning- P=0.086, Episodic memory- P=0.097, Global cognition- P=0.054).

Table 3.15 Relationship between education and cognitive function in the old adults, stratified by gender

		Basic School		High School		University		
		n	Z-Score	n	Z-Score	n	Z-Score	*P*
Male								
	Executive functioning	14	-3.123	23	-2.270	49	-2.084	0.226
	Working memory capacity	14	-1.793	23	-1.718	49	-1.767	0.993
	Episodic memory	14	-4.483	23	-1.878	49	-2.970	0.119
	Global cognition	14	-9.403	23	-5.866	49	-6.821	0.106
Female								
	Executive functioning	19	-3.001	47	-2.965	33	-2.151	0.089
	Working memory capacity	19	-2.454	47	-2.060	33	-1.668	**0.001**
	Episodic memory	19	-5.148	47	-2.419	33	-2.210	**0.015**
	Global cognition	19	-10.603	47	-7.444	33	-6.030	**0.023**

P values were determined by independent samples Kruskal-Wallis H tests

3.11 Relationship between education and plasma BDNF levels, in the old adults

With the old adults displaying improved cognitive function with further progression of education, we investigated whether plasma BDNF levels were attributable to this difference.

In the old adults, an increase of BDNF plasma protein trended (P=0.066) with the further the participants had progressed in their education, though no significant differences were seen in the stratified gender groups (Table 3.16).

Table 3.16 Relationship between education and BDNF plasma protein, stratified by gender

	Basic School		High School		University		
BDNF plasma (Z-Score)	n	Z-Score	n	Z-Score	n	Z-Score	*P*
Male	14	0.437	23	0.748	49	0.820	0.387
Female	19	0.156	46	0.105	27	0.357	0.572
Total	33	0.275	69	0.319	76	0.655	0.066

P values were determined by independent samples Kruskal-Wallis H tests

3.12 Relationship between education and blood *BDNF* methylation, in the old adults

In the old adults, it was observed that *BDNF* exon IV had reduced methylation at CpG sites 3 and 4 in those who had progressed further into education, however, while there were reductions across the site, no other trend was close to being significant. *BDNF* exon I showed no changes between the groups (Table 3.17).

Further analysis between the individual groups of basic and university education, showed a significant reduction in *BDNF* exon IV methylation in those with a university education (Mann Whitney U: CpG 3- P=0.026, CpG 4- P=0.018, CpG 5- P=0.022, Average methylation- P=0.035).

Table 3.17 Relationship between education and *BDNF* methylation, in the old adults

		Basic School		High School		University		
		n	Methylation	n	Methylation	n	Methylation	*P*
BDNF Exon I								
	CpG 1	15	0.400	36	0.263	25	-0.077	0.173
	CpG 2	15	0.533	36	0.788	25	0.166	0.442
	CpG 3	15	0.641	36	0.905	25	0.450	0.587
	CpG 4	15	0.605	36	0.885	25	0.592	0.810
	CpG 5	15	0.420	36	0.823	25	0.486	0.764
	Average	15	0.725	36	1.307	25	0.543	0.781
BDNF Exon IV								
	CpG 1	12	0.235	32	-0.004	29	-0.252	0.595
	CpG 2	12	0.384	32	-0.256	29	-0.170	0.278
	CpG 3	12	0.579	32	-0.025	29	-0.479	**0.040**
	CpG 4	12	0.675	32	-0.295	29	-0.573	**0.045**
	CpG 5	12	0.470	32	-0.353	29	-0.407	0.080
	CpG 6	12	0.261	32	-0.036	29	-0.092	0.733
	CpG 7	12	0.451	32	-0.354	29	-0.337	0.177
	CpG 8	12	0.119	32	-0.149	29	-0.449	0.670
	CpG 9	12	0.555	32	0.071	29	-0.313	0.417
	Average	12	0.642	32	-0.080	29	-0.307	0.135

BDNF methylation is presented as a percentage, with Z-Scores based of the average of the young male and female populations separately. P values were determined by independent samples Kruskal-Wallis H tests.

3.13 Relationship between *BDNF* Val66Met genotype and cognition

This study observed that the Val66Met polymorphism influenced cognitive function in the male population, in which the methionine substitution (AA/AG) was negatively associated with cognitive scores (Table 3.18). The negative association shown in the male population was preserved after controlling for age in working memory capacity and global cognition (Table 3.19, Figure 3.12).

Table 3.18 Relationship between Val66Met genotype and cognitive function, stratified by gender.

	Male			Female		
	Val vs Met substitution			Val vs Met substitution		
	GG	AA/AG	*P*	GG	AA/AG	*P*
Executive functioning	-1.344	-2.183	**0.016**	-1.789	-1.566	0.489
Working memory capacity	-0.836	-1.571	**0.001**	-1.188	-1.328	0.646
Episodic memory	-1.740	-3.464	0.112	-1.742	-2.162	0.125
Global cognition	-3.920	-7.217	**0.003**	-4.719	-5.056	0.617

P values were determined by independent samples Mann-Whitney U tests

Table 3.19 Relationship between val66met genotype and cognitive function

	Male	Female	Total
	P	*P*	*P*
Executive functioning	0.152	0.118	0.857
Working memory capacity	**0.004**	0.849	**0.044**
Episodic memory	0.087	0.693	0.129
Global cognition	**0.018**	0.865	0.137

P values were determined by individual ANCOVAs, with age as a covariant

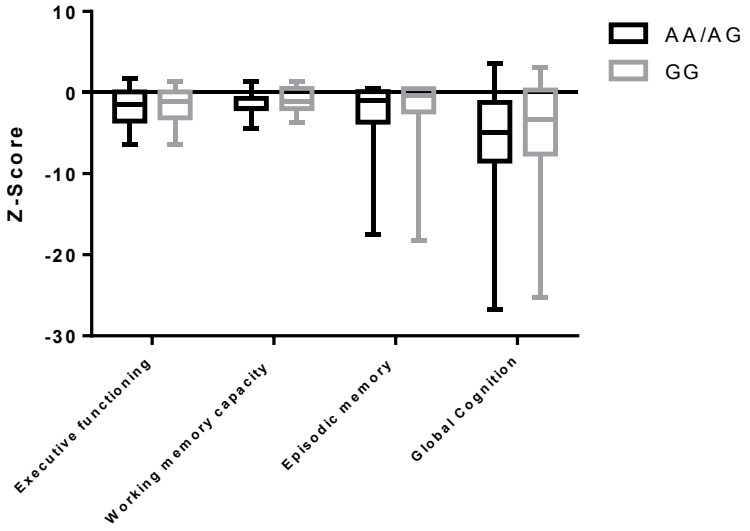

Figure 3.12. Relationship between *BDNF* Val66Met and cognitive function. Working memory capacity and global cognition were significantly higher in those who were GG homozygous (P=0.004 and P=0.018, respectively). Episodic memory was marginally higher in those who were GG homozygous (P=0.087). No significant difference was observed in executive functioning.

To determine whether the Val66Met polymorphism had a specific influence on cognitive ageing, the study further stratified by age (Table 3.20). Notably, the trend of reduced cognitive function with the methionine substitution remained in the old male adults, with a significant fall in working memory capacity (P=0.034).

Table 3.20 Relationship between Val66Met genotype and cognitive function, stratified by age and gender.

		Male			Female		
		Val vs Met substitution			Val vs Met substitution		
		GG	AA/AG	*P*	GG	AA/AG	*P*
Young							
	Executive functioning	0.210	0.001	0.197	-0.087	0.094	0.625
	Working memory capacity	0.270	-0.176	0.080	0.019	-0.221	0.295
	Episodic memory	0.033	0.157	0.458	0.166	-0.069	0.271
	Global cognition	0.513	-0.018	0.099	0.098	-0.196	0.538
Old							
	Executive functioning	-2.510	-2.911	0.214	-3.015	-2.464	0.149
	Working memory capacity	-1.666	-2.035	**0.034**	-2.057	-1.926	0.465
	Episodic memory	-3.069	-4.671	0.475	-3.117	-3.293	0.402
	Global cognition	-7.245	-9.617	0.117	-8.189	-7.683	0.690

P values were determined by independent samples Mann-Whitney U tests

3.14 Relationship between *BDNF* Val66Met genotype and plasma BDNF levels

When the BDNF protein in the plasma was compared between valine and methionine substitutions, no significant difference was observed in any group (Table 3.21, Figure 3.13).

Table 3.21 Relationship between val66met genotype and BDNF plasma protein

	Male			Female			Total		
	Val vs Met substitution			Val vs Met substitution			Val vs Met substitution		
BDNF plasma (Z-Score)	GG	AA/AG	*P*	GG	AA/AG	*P*	GG	AA/AG	*P*
Young	-0.071	0.252	0.451	0.030	0.068	0.779	0.002	0.149	0.454
Old	0.664	0.937	0.521	0.096	0.350	0.453	0.364	0.634	0.211
Total	0.381	0.788	0.157	0.070	0.266	0.444	0.218	0.498	0.088

P values were determined by independent samples Mann-Whitney U tests

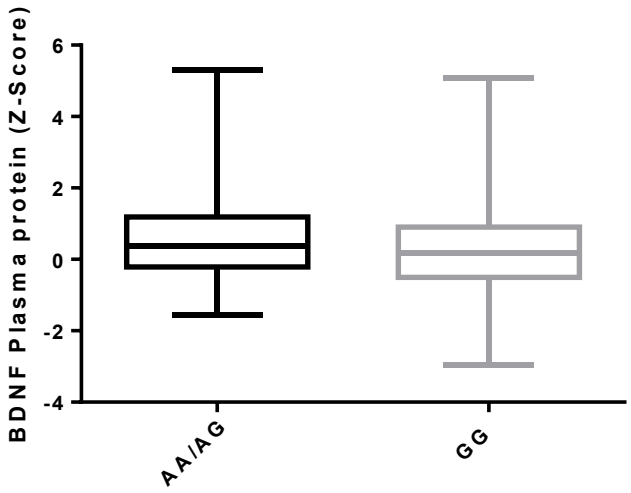

Figure 3.13. Relationship between Plasma BDNF and *BDNF* Val66Met.
No significant difference was observed between the genotypes.

3.15 Regulation of *BDNF* gene expression in the prefrontal cortex with age

To gain a clearer understanding of the control of BDNF and its role in cognition we next focused on testing its regulation within the central nervous system, where BDNF is most biologically active. Therefore, this study tested brain material from the Dyne-Steele cohort, which had donated brains from participants who had been longitudinally studied for changes in cognitive function for around 30 years. Brains from all those participants who had died were studied for neuropathological changes revealing that they had passed away from AD related pathology or from causes that have no link to neurodegeneration (controls).

While this cohort exclusively took samples from an aged population (>70 years old), the range of ages when the samples were taken still varied by 30 years (72-104 years old). Thus, *BDNF* gene expression from prefrontal cortex was correlated across the age of the participants upon death, controlling for neuropathology. It was observed that *BDNF* exon IV reduced significantly with age, while total *BDNF* expression showed a similar weaker reduction (Table 3.22, Figure 3.14).

Table 3.22 Relationship between age and *BDNF* expression

	Age at death	
	r	*P*
BDNF exon I expression	-0.216	0.135
BDNF exon IV expression	-0.336	**0.018**
BDNF total expression*	-0.228	0.075

*r= Pearson correlation coefficient. *Spearman correlation*

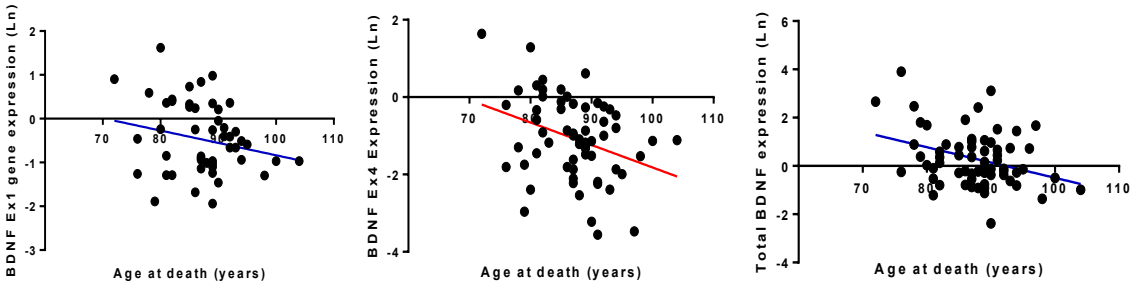

Figure 3.14. Relationship between BDNF expression and age. Left) Relationship between *BDNF* exon I expression and age, no significant association was observed. Middle) Relationship between *BDNF* exon IV expression and age, *BDNF* exon IV expression declined significantly with age (P=0.018). Right) Relationship between total *BDNF* expression and age, total *BDNF* expression declined marginally with age (P=0.075).

The expression of *BDNF* exon I varied significantly with gender (Female: -0.725, Male: 0.146, P<0.001). Therefore, the analysis of *BDNF* exon I expression against age at death was split by gender, while controlling for neuropathology (Table 3.23). Although, no significant association was observed between age and *BDNF* exon I expression, in either males or females.

Table 3.23 Relationship between age and *BDNF* exon I expression, stratified by gender

	Age at death			
	Male		Female	
	r	*P*	*r*	*P*
BDNF exon I expression	-0.392	0.133	-0.254	0.130

r= Pearson correlation coefficient.

3.16 Relationship between *BDNF* gene expression in the prefrontal cortex and cognition

It was observed that *BDNF* exon I expression was negatively associated with baseline performances in memory and speed as well as being associated with a faster age-related decline in memory (Table 3.24). Even after controlling for age and neuropathology, *BDNF* exon I expression was still significantly associated with poorer memory scores and a faster rate of decline (ANCOVA: cross-sectional memory- β=-0.302, P=0.035; longitudinal memory- β=-0.388, P=0.008).

Table 3.24 Relationship between *BDNF* expression and cognitive function

	BDNF exon I expression		*BDNF* exon IV expression		*BDNF total* expression	
	r	*P*	*r*	*P*	*R**	*P*
Cross-sectional fluid intelligence	-0.234	0.092	-0.189	0.135	-0.039	0.754
Cross-sectional vocabulary	-0.149	0.287	0.028	0.825	-0.224	0.071
Cross-sectional speed	-0.313	**0.024**	-0.257	**0.044**	-0.077	0.547
Cross-sectional memory	-0.347	**0.012**	0.013	0.918	-0.187	0.136
Longitudinal fluid intelligence	-0.120	0.394	-0.128	0.313	-0.011	0.933
Longitudinal vocabulary	-0.164	0.241	0.009	0.945	-0.235	0.058
Longitudinal speed	-0.206	0.138	-0.204	0.105	0.073	0.563
Longitudinal memory	-0.385	**0.004**	-0.060	0.639	-0.194	0.118

r= Pearson correlation coefficient. R= Spearman correlation coefficient*

We analysed *BDNF* expression against cognitive tests, in the healthy ageing group and AD group, separately, while controlling for age at cognitive testing (Table 3.25). In the healthy ageing group, *BDNF* exon IV expression was weakly associated with improved baseline memory and slower decline of memory. The opposite was observed in the AD group, as *BDNF* exon I and total *BDNF* expression were associated with impaired memory and a faster rate of decline in memory function.

Table 3.25 Relationship between *BDNF* expression and cognition, stratified by Alzheimer's Disease

	BDNF exon I expression		*BDNF* exon IV expression		*BDNF total* expression	
	r	*P*	*r*	*P*	*r**	*P*
Age changes only						
Cross-sectional fluid intelligence	-0.016	0.959	-0.087	0.779	0.139	0.634
Cross-sectional vocabulary*	0.230	0.430	0.243	0.403	0.151	0.606
Cross-sectional speed	0.171	0.576	0.318	0.290	0.104	0.722
Cross-sectional memory	0.232	0.445	0.548	**0.053**	0.042	0.887
Longitudinal fluid intelligence	-0.069	0.823	-0.078	0.801	0.076	0.797
Longitudinal vocabulary*	0.221	0.448	0.244	0.401	0.154	0.599
Longitudinal speed	0.110	0.720	0.282	0.351	0.432	0.123
Longitudinal memory	0.030	0.923	0.506	0.078	0.091	0.756
AD						
Cross-sectional fluid intelligence	-0.206	0.428	0.102	0.746	-0.111	0.670
Cross-sectional vocabulary*	0.162	0.534	0.461	0.062	-0.103	0.694
Cross-sectional speed	-0.206	0.427	0.153	0.495	-0.219	0.399
Cross-sectional memory	-0.551	**0.022**	-0.174	0.132	-0.558	**0.020**
Longitudinal fluid intelligence	-0.051	0.845	0.142	0.926	0.056	0.832
Longitudinal vocabulary*	0.085	0.746	0.375	0.138	-0.254	0.325
Longitudinal speed	-0.077	0.769	0.206	0.665	-0.018	0.947
Longitudinal memory	-0.618	**0.008**	-0.362	0.153	-0.458	0.065

*r= Pearson partial correlation coefficient. *=Spearman partial correlation.*

3.17 Relationship between prefrontal cortex *BDNF* gene expression and neuropathology

The average expression for each neuropathology group is displayed in Table 3.26. Analysis of *BDNF* expression between neuropathology groups found significant differences in *BDNF* exon I expression between the BRAAK groups (P=0.045), however post hoc analysis found no significant differences between the individual groups. There was also observed to be significant differences in *BDNF* total expression between the THAL groups (P=0.036), however post-hoc analysis showed no significant differences between the groups (Figure 3.15).

Table 3.26 Relationship between neuropathology and *BDNF* expression

	BDNF exon I expression		*BDNF* exon IV expression		*BDNF* total expression	
	n	log	n	log	n	log
THAL Group						
0	14	-0.363	17	-1.164	17	0.713
1	9	-0.853	10	-1.009	10	-0.472
2	6	-0.155	6	-0.840	6	-0.176
3	11	-0.243	15	-1.010	17	0.676
4	7	-0.624	9	-1.162	9	0.047
5	6	-0.623	7	-1.026	7	0.160
P		0.479		0.991		**0.036**
CERAD Group						
0	15	-0.399	18	-1.102	18	0.690
1	16	-0.489	18	-0.822	18	-0.131
2	14	-0.422	17	-1.249	18	0.153
3	8	-0.593	11	-1.077	12	0.548
P		0.952		0.699		0.252
BRAAK Group						
0	3	-0.173	4	-1.126	4	0.929
1	24	-0.648	28	-1.104	28	0.144
2	21	-0.179	24	-0.864	25	0.294
3	4	-1.211	7	-1.492	8	0.584
P		**0.045**		0.582		0.697

BDNF gene expression is presented as log. Neuropathology groups were previously devised from neuropathology scores. P values for BDNF exon I and BDNF exon IV expression were determined using One-Way ANOVAs. P values for BDNF total expression were determined using Kruskal Wallis H tests

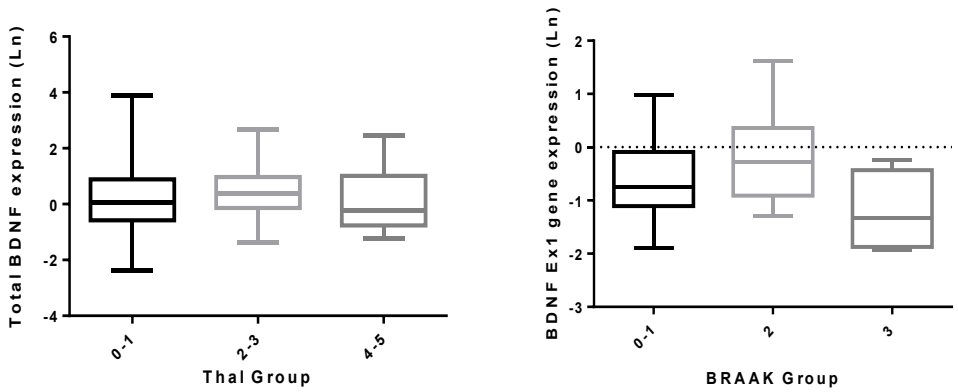

Figure 3.15. Relationship between BDNF expression and neuropathology. Left) Relationship between total *BDNF* expression and neurofibrillary tangle accumulation, total *BDNF* expression exhibits a minimal increase to neurofibrillary tangle accumulation, but decreases with further accumulation. Right) Relationship between *BDNF* exon I expression and β-amyloid accumulation, BDNF exon I expression increases in response to initial accumulation, but decreases with higher β-amyloid load.

3.18 Correlation between methylation at individual CPG sites within promoters I and IV of the *BDNF* gene in the prefrontal cortex

In the region analysed within the *BDNF* exon I promoter, the methylation of all the CpG sites were significantly correlated with one another (Figure 3.16, left, r>0.380, p<0.005). However, within the *BDNF* exon IV promoter region (Figure 3.16, right), there was no consistent pattern of correlation between the CpG sites. Therefore, an average methylation score could only be made for the *BDNF* exon I region. *BDNF* methylation was lower in the Dyne-Steele cohort than the MyoAge cohort (Table 2.1; 2.2, Methods).

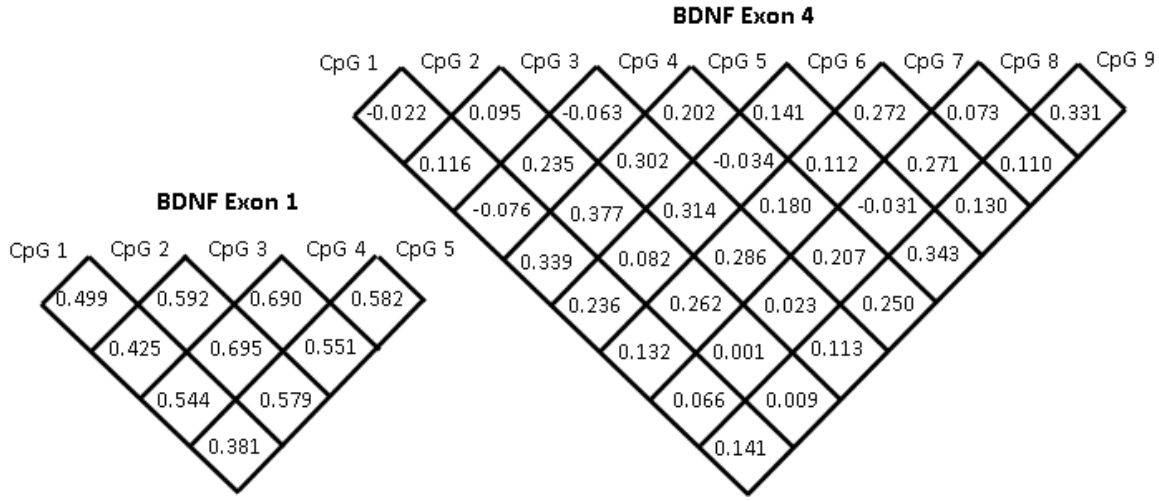

Figure 3.16. Spearman's correlation between CpGs in BDNF exon I (left) and IV (right). All correlations within *BDNF* exon I were significant (p<0.005)

3.19 Relationship between *BDNF* methylation and *BDNF* expression in the prefrontal cortex

It was observed that methylation across the analysed *BDNF* exon I promoter was negatively associated with *BDNF* exon I gene expression (Table 3.27). No other associations were observed between methylation of the *BDNF* exon I or 4 regions and gene expression (Figure 3.17). When the healthy ageing group and AD group were analysed separately, *BDNF* exon I methylation showed a weak negative association with *BDNF* exon I gene expression, though no associations were observed in the AD group

Table 3.27 Relationship between *BDNF* methylation and BDNF expression

		BDNF exon I expression		*BDNF* exon IV expression		*BDNF* total expression	
		r	P	r	P	r	P
BDNF exon I							
	CpG 1	-0.060	0.685			-0.128	0.333
	CpG 2	-0.176	0.212			-0.145	0.251
	CpG 3	-0.230	0.105			-0.217	0.085
	CpG 4	-0.185	0.188			-0.007	0.959
	CpG 5	-0.021	0.885			-0.091	0.482
	Average*	-0.273	**0.051**			-0.190	0.130
BDNF exon IV							
	CpG 1			0.006	0.964	0.124	0.324
	CpG 2			-0.004	0.974	-0.195	0.143
	CpG 3			-0.177	0.161	0.071	0.574
	CpG 4			0.004	0.973	-0.171	0.173
	CpG 5			0.162	0.201	-0.200	0.111
	CpG 6			-0.067	0.639	0.159	0.261
	CpG 7			-0.049	0.698	0.121	0.336
	CpG 8			0.008	0.951	0.077	0.542
	CpG 9			-0.018	0.885	-0.205	0.101

r= Spearman correlation coefficient. r=Average methylation against BDNF exon I expression was tested using Pearson correlation*

(Table 3.28).

Table 3.28 Relationship between *BDNF* exon 1 methylation and BDNF expression, stratified by Alzheimer's Disease

	BDNF exon I expression		*BDNF* total expression	
	r*	P	r	P
Healthy Ageing				
BDNF exon I Average	-0.474	0.074	-0.233	0.385
Alzheimer's Disease				
BDNF exon I Average	-0.315	0.189	-0.044	0.826

*r= Spearman correlation coefficient. *Pearson correlation*

3.20 Relationship between *BDNF* methylation in the prefrontal cortex and cognition

This study observed no significant associations between *BDNF* methylation and cognitive function, while controlling for age and neuropathology, in the Dyne-Steele cohort (Table 3.29), although a weak association was observed between *BDNF* exon I methylation and faster decline of memory (Figure 3.18).

Table 3.29 Relationship between *BDNF* methylation and cognitive function

	Cross-sectional fluid intelligence	Cross-sectional vocabulary	Cross-sectional speed	Cross-sectional memory	Longitudinal fluid intelligence	Longitudinal vocabulary	Longitudinal speed	Longitudinal memory
	r	r	r	r	r	r	r	r
BDNF exon I								
CpG 1	0.005	0.077	-0.157	**-0.307**	0.024	0.047	-0.108	-0.136
CpG 2	0.082	0.087	0.180	**-0.279**	0.129	0.106	0.271	-0.209
CpG 3	0.057	0.177	-0.020	0.021	0.084	0.188	0.038	-0.017
CpG 4	0.161	0.179	0.058	-0.046	0.188	0.177	0.063	-0.073
CpG 5	0.143	0.088	0.103	-0.172	0.086	0.067	0.202	-0.243
Average*	0.047	0.017	0.044	-0.225[#]	0.093	0.011	0.056	-0.189[#]
BDNF exon IV								
CpG 1	-0.135	-0.205	-0.048	0.098	-0.115	-0.208	-0.123	0.064
CpG 2	-0.053	0.041	-0.081	-0.069	-0.074	-0.007	-0.001	-0.162
CpG 3	-0.196	-0.053	**-0.331**	-0.142	-0.091	-0.093	-0.229	-0.077
CpG 4	-0.161	-0.001	-0.069	0.175	-0.065	0.013	-0.081	0.073
CpG 5	-0.088	0.080	-0.110	0.183	-0.106	0.033	-0.207	0.123
CpG 6	0.021	0.129	0.300	0.141	0.032	0.098	0.318	0.141
CpG 7	-0.016	-0.115	-0.148	-0.083	0.047	-0.157	-0.200	-0.105
CpG 8	0.077	0.072	0.258	0.097	0.100	0.132	0.280	0.183
CpG 9	0.019	0.258	0.207	0.205	0.115	0.223	0.100	**0.335**

*r= Spearman partial correlation coefficient. *Average methylation was tested using Pearson partial correlation. Highlighted correlations were significant at 0.05 level (P<0.05). No significance was observed in individual CpG sites after adjusting for multiple analyses. # BDNF exon I average methylation was weakly associated with cross-sectional memory (P=0.068) and longitudinal memory (P=0.065).*

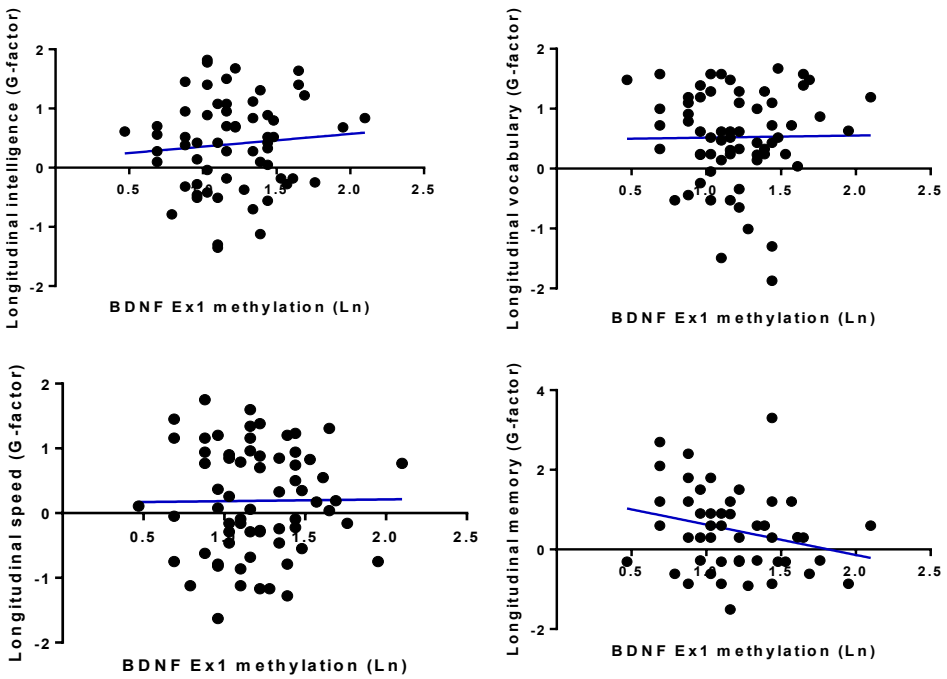

Figure 3.18. Relationship between BDNF exon I methylation and longitudinal cognitive function. Top left) Relationship between *BDNF* exon I methylation and longitudinal fluid intelligence. Top right) Relationship between *BDNF* exon I methylation and longitudinal fluid vocabulary. Bottom left) Relationship between *BDNF* exon I methylation and longitudinal speed. Bottom right) Relationship between *BDNF* exon I methylation and longitudinal memory. No significant associations were observed, although increase methylation was weakly associated with a faster decline of memory (P=0.065).

We stratified the population between healthy ageing and AD, and reanalysed *BDNF* exon I methylation against cognitive function, controlling for age and neuropathology (Table 3.30). No significant associations were observed in either group.

Table 3.30 Relationship between *BDNF* exon I methylation and cognitive function, stratified by Alzheimer's disease

Cross-sectional fluid intelligence	Cross-sectional vocabulary	Cross-sectional speed	Cross-sectional memory	Longitudinal fluid intelligence	Longitudinal vocabulary	Longitudinal speed	Longitudinal memory
r	r	r	r	r	r	r	r
Healthy ageing							
0.164	0.053	-0.217	-0.214	-0.053	0.089	-0.055	-0.089
Alzheimer's Disease							
0.031	-0.164	0.045	-0.237	-0.075	-0.200	0.062	-0.240

r= Pearson partial correlation coefficient. No correlations were significant at 0.05 level (P<0.05).

3.21 Regulation of H3K27 acetylation at *BDNF* exon IV in the prefrontal cortex

In light of the lack of association between *BDNF* exon IV methylation and *BDNF* expression in the brain, we considered an alternative epigenetic mark, histone H3K27 modification. Specifically, we wanted to determine whether functional polymorphisms around the *BDNF* exon IV promoter affected the binding of H3K27Ac to the region.

The rs56133711 polymorphism was considered the most informative choice based on its proximity to the *BDNF* exon 4 promoter and known H3K27Ac binding sites (Figure 1.5; Figure 3.19), as well as having a relatively high minor allele frequency (approximately 20% in the European population). It is characterised by a G/A conversion approximately 150 bp upstream of the *BDNF* exon IV.

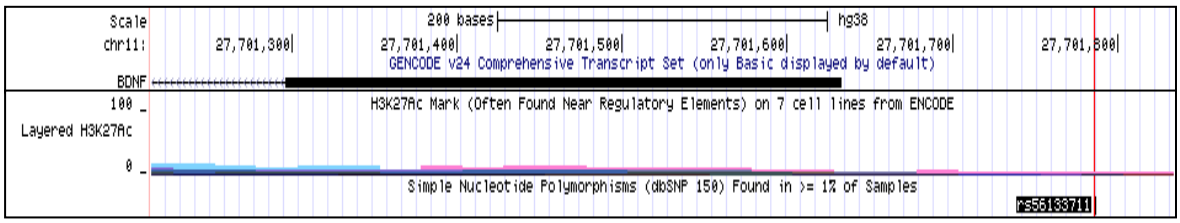

Figure 3.19. BDNF exon IV and rs56133711. The rs56133711 is 154bp upstream of *BDNF* exon IV and is located in a H3K27Ac binding region.

We performed allele-specific ChIP analysis in brain to determine whether rs56133711 differently associates with H3K27Ac (Figure 3.20). ChIP qPCR results showed that H3K27Ac was binding to *BDNF* exon IV (Figure 3.21).

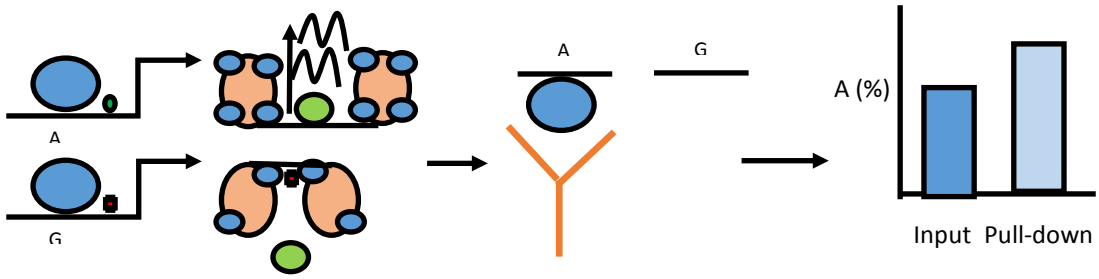

Figure 3.20. Schematic of ChIP allele discrimination principles. Left) Allele discrimination. Histone H3K27ac is associated with an active chromatin state, therefore if the histone H3K27ac marker more readily binds to one of the rs56133711 alleles, then that allele could be associated with higher *BDNF* expression. Middle) Summary of ChIP (Murgatroyd et al, 2012). Anti-H3K27ac antibody binds to the H3K27ac marker, along with the sheared DNA that it is bound to. Immune complexes are cleaned, leaving only the bound DNA in the pull-down sample. Left) Results from sequencing rs56133711 heterozygotes. Input should be 50/50 A/G, however if allele discrimination is present, then the pull-down sample will be higher or lower than 50%.

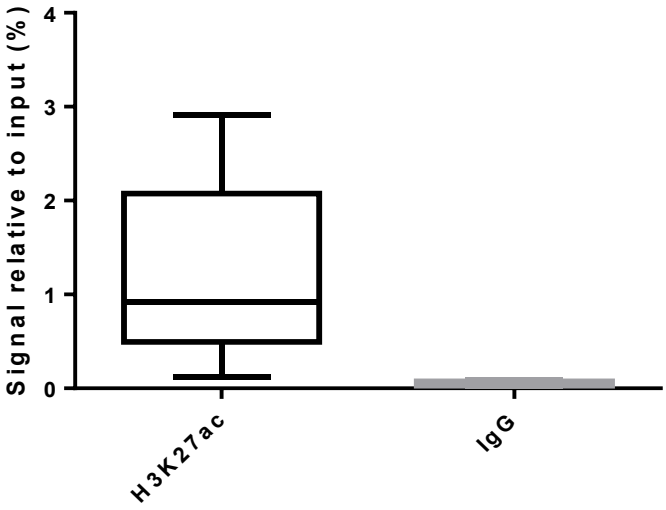

Figure 3.21. Results of ChIP qPCR. The higher signal relative to input of the H3K27ac pull-down in contrast to next to no signal displayed in the IgG sample, showed that H3K27ac was binding to the rs56133711 region, while the IgG was not.

Testing the ChIP pulldown with the different alleles it was observed that the H3K27Ac mark more readily interacted with the A allele than the G allele (Figure 3.22, Alternate strand sequenced: T nucleotide- input= 49.63%, pull-down= 72.25%, P=0.055).

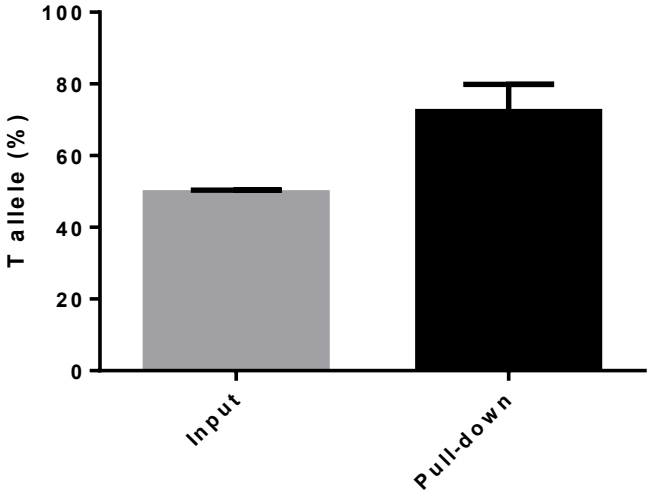

Figure 3.21. *BDNF* rs56133711 allele discrimination. A higher amount of T alleles were sequenced from the ChIP pull-down than the input samples (P=0.055), suggesting H3K27ac more actively binds to the rs56133711 A polymorphism.

3.22 Relationship between *BDNF* rs56133711 polymorphism and *BDNF* expression in brain

With initial results showing that an interaction between rs56133711 and chromatin binding, we analysed whether this polymorphism related to expression of *BDNF.* However, when testing RNA levels across the brains with genotypes no significant association was seen between *BDNF* exon IV or *BDNF* total expression and the different alleles of *BDNF* rs56133711 (Table 3.31).

Table 3.31 Relationship between *BDNF* rs56133711 and *BDNF* gene expression

	rs56133711 allele			
	AA (n=4)	AG (n=27)	GG (n=36)	*P*
BDNF exon IV expression	-0.159	-1.280	-0.994	0.127
BDNF total expression	0.318	0.342	0.222	0.868

P values were determined by a one-way ANOVA(*BDNF* exon IV) and a Kruskal-Wallis H test (*BDNF total)*

4. Discussion

This study set out to analyse the different regulatory mechanisms that play a role in the expression of BDNF, with a focus on epigenetic regulation. Specifically, the aim was to determine whether these regulatory mechanisms are involved in cognitive ageing; observing the changes in DNA methylation with age, while deducing if these changes are contributing to reduced expression of BDNF, and subsequent cognitive decline. Moreover, this study compared the impact of various lifestyle factors on DNA methylation, determining whether both short and long term epigenetic changes were impacting the susceptibility of the population to age-related cognitive decline. It was also investigated how genetic variation in the *BDNF* gene could impact the regulation of BDNF, while determining if there were any downstream cognitive changes. Finally, we intended to analyse the interaction between the genetic and epigenetic mechanisms involved in the *BDNF* gene, whether there is any in the first instance, and if so whether it is attributable to cognitive function and ageing.

4.1 Relationship between plasma BDNF and age

Unexpectedly, BDNF levels measured in the plasma were significantly higher in the old adults than the young adults. With gender previously being reported as a potential cofounder of BDNF protein levels (Bus et al, 2011; Erickson et al, 2010), the population was stratified and it was revealed that the old male adults had significantly higher plasma BDNF protein than the young males, while there were no significant differences in the female population (Figure 3.2). A large body of research that had investigated the role of BDNF in cognitive ageing made this result surprising, especially since previous research has shown peripheral BDNF levels to decline with age (Lommatzsch et al, 2005; Ziegenhorn et al, 2007; Leckie et al, 2014; Shimada et al 2014). A previous study did describe a rise in peripheral BDNF with age (Bus et al, 2011), however it was found in the female population rather than the male population. Notably, all the examples of declining BDNF with age are derived from serum BDNF rather than plasma BDNF, which was used in this study. A recent study by Polacchini et al (2015) summarised the current methods for measurement of serum BDNF, noting that BDNF measurements are of limited value until reliable and replicable results can be produced. Furthermore, they suggested that

serum BDNF is potentially more reliable than plasma BDNF, supported by the observation that serum has a 100-fold higher concentration of BDNF than plasma (Radka et al, 1996). While this study measured relatively high plasma BDNF levels in comparison to the levels reported, they were still approximately 10-fold lower than the serum levels recorded in the previous studies. It has also been stated that due to BDNF protein being stored and secreted from platelets (Fugimura et al, 2002), the reproducibility of measurements could prove difficult, as simply inserting a needle into the plasma could trigger platelets to release stored BDNF.

4.2 Relationship between plasma BDNF and cognitive function

Due to the consideration that the clear majority of previous research has encountered a decline in serum BDNF with age, as well as the previous discovery that the MyoAge cohort displayed a negative correlation in cognitive function with age (Bradburn et al, 2016), the study analysed whether BDNF was potentially attributable to cognitive ageing.

No significant correlations were found between plasma BDNF levels and any of the cognitive functions in the MyoAge population (Table 3.1). When the population was further stratified by age, there was an observation that plasma BDNF levels were positively correlated with MMSE scores in the old females (Table 3.2). The MMSE scores were then shown to be positively associated with the cognitive scores obtained in the MyoAge cohort, an association that was also preserved specifically within the old female population. While the MMSE score represents a more general analysis of cognitive function than the CANTAB tests, it is capable of registering phenomena such as cognitive ageing and monitor neurological disease progression (Woodford and George, 2007). Similar tests are used to research cognition in various populations and have previously been associated with BDNF levels (Zou et al, 2014).

A lack of association with between BDNF and cognitive function overall, as well as within sexes, weakens the hypothesis that BDNF could mediate age-related changes in cognitive functions. Despite this, BDNF levels appear to be protective against cognitive decline within the old female population, displayed by the positive association within the group. There is a profound amount of data on the positive role of BDNF on cognitive function (Croll et al, 1998; Egen et al, 2003; Blurton-Jones et al, 2009), which has led to the

hypothesis that BDNF is protective in age-related cognitive decline. This study suggests a gender specific role for BDNF as a protective mechanism against cognitive ageing, an observation that has been supported previously (Komulainen et al, 2008). Although no specific BDNF mediation on cognitive ageing was observed, it has been observed elsewhere (Erickson et al, 2010) and therefore, further research into this association and the mechanisms behind it are warranted.

4.3 Relationship between blood *BDNF* methylation and age

One such mechanism is *BDNF* methylation (McKinney et al, 2015). This study demonstrated that there was a highly significant increase in methylation at the promoter region of *BDNF* exon I with increasing age (Table 3.4). This was analysed further to find that the significant increase in methylation was only found in the old females compared to the young females, although the trend was also seen in the male population (Table 3.5). It is known that DNA methylation varies with age, and that the variation depends upon the region of DNA, tissue source of DNA and the condition of the participants that DNA was collected from (Fraga et al, 2009; Marioni et al, 2015). Two interesting points of research have been published that have found that there is some correlation between *BDNF* methylation in the peripheral tissues and *BDNF* methylation in the hippocampus (Kundakovic et al, 2015) and the prefrontal cortex (Stenz et al, 2015). With regards to *BDNF* methylation, the picture is not as clear as expected, primarily due to much of the research being focused on neuropsychological and neurodegenerative diseases, specifically. This only enhances the importance of understanding the role of disease free ageing on *BDNF* methylation, as adequate healthy controls should be referred to when observing disease models. McKinney et al (2015) have previously shown that methylation of *BDNF* was higher in brains from older individuals than from younger individuals, supporting the data of this study, and presenting a model for *BDNF* methylation as a mechanism of age-related decline in BDNF expression.

4.4 Relationship between *BDNF* methylation, *BDNF* expression and plasma BDNF levels

No significant correlations were observed between *BDNF* exons I or IV and plasma BDNF levels in either males or females (Tables 3.6; 3.7). As previously stated, any age-

associated changes in this cohort were not expected to be found due to the unexpected relationship shown between plasma BDNF and age (refer to section 4.1), although this further suggests that methylation at the promoter regions does not regulate peripheral BDNF levels. This does not rule out *BDNF* methylation as a regulator of plasma BDNF protein. Due to the crucial function and expression of BDNF across multiple tissues, there are nine functional promotors within the *BDNF* gene (Pruunsild et al, 2007), which through alternative splicing produce numerous transcripts. The different transcripts may be generated via varying cellular stimuli, and as a result may be localised and more stable within specific cells and sub-cellular compartments (Pruunsild et al, 2007; Cattaneo et al, 2016). Cattaneo et al (2016), compared the expression of 12 of the main *BNDF* transcripts between peripheral blood cells and the hippocampus, where they found that transcripts including I, Va, Vb and VIIb were expressed in low levels in the peripheral blood, in comparison to the hippocampus. On the other hand, *BDNF* transcripts IV, IXabd and IXabcd were highly expressed in peripheral blood cells and the hippocampus. As CGIs are more frequently located upstream or around promoters, as observed with exons I and IV, *BDNF* methylation is still likely to contribute to the variation in transcript expression between tissues. This is supported by previous research that has displayed significant between-tissue variation in DNA methylation, including within the *BDNF* gene (Davies et al, 2012), although this is predominantly found in intragenic CGIs rather than promoter CGIs (Deaton et al, 2011; Davies et al, 2012).

The function of intragenic DNA methylation is still controversial, as various reports have described its role in gene activation (Flanagan et al, 2007; Ball et al, 2009), as it may inhibit antisense transcripts that generate RNAi complementary to mRNA molecules. Alternatively, it may have a role in gene suppression, as Maunakea et al (2010) found that like promoter CGIs, intra- and inter-genic CGIs had CAGE clusters mapped to them, which are indicative of transcription start sites (Carninci, 2006), and therefore theorised that intragenic CGIs may in fact regulate alternative gene promoters. Further characterisation of intragenic methylation is required to elucidate its role within gene expression.

Further analysis is recommended into the relation between *BDNF* methylation at the different *BDNF* exons and the *BDNF* transcripts, between tissues. As Cattaneo et al (2016) found, there appears to be more expression of *BNDF* transcripts IV, IXabd and IXabcd, and

so further methylation analysis should be carried out across the promoter and intragenic CGIS near to exons IV and IX. Even while this study failed to find an association with *BDNF* exon IV and plasma BDNF levels, a more widespread analysis than the region selected may provide a different picture.

Variations in intragenic methylation between tissue types (Davies et al, 2012) appears to counteract the observations made by Kundakovic et al (2015) and Stenz et al (2015). This presents the immediate question: does *BDNF* methylation in the peripheral tissues predict biological changes in the brain as this author has previously suggested? With regards to Davies et al (2012), while it may be that there are tissue differences in methylation between most intragenic CGIs, promoter CGI methylation remained relatively conserved. Furthermore, it was noted that although it was mainly outweighed by between tissue variation, between individual variation in DNA methylation was correlated between brain and blood, suggesting that peripheral methylation might still be useful as a biomarker for the changes seen in some neurological conditions. Since *BDNF* methylation at promoters has been shown to inhibit gene expression (Klose and Bird, 2006; Cedar and Bergman, 2009), between individual variation occurring at these sites could present a clear pathway for peripheral methylation to mirror CNS methylation, highlighting changes in expression within the brain. This could also be said of intragenic methylation once more is understood about its function. Peripheral *BDNF* methylation in particular may be more relevant to neurological function, as BDNF actively crosses the BBB (Poduslo et al, 1996; Pan et al, 1998). Even though this is a two-way flow, it suggests that BDNF generated peripherally contributes at least in some way to BDNF within the brain.

4.5 Relationship between blood *BDNF* methylation and cognitive function

It is because of these reasons that we studied the role of *BDNF* methylation as a biomarker of cognitive function. This study observed a significant negative association between *BDNF* exon I methylation and executive functioning, with further weaker negative associations with working memory capacity and global cognition (Table 3.8). It appears that this negative association is attributable predominantly to the male population, with no associations observed within the female population (Table 3.9). In

addition to the association across the total male population, it was found that *BDNF* exon I methylation had a strong negative correlation with executive functioning and working memory capacity within the old male population (Table 3.10). These both suggest that DNA methylation is having a gender specific effect on the *BDNF* gene which is impacting cognitive function. Specifically, we theorise that *BDNF* methylation at exon I is reducing the generation of the *BDNF* exon I transcript, leading to a reduction in BDNF expression. This is hampering the ability of BDNF to maintain neuroplasticity required for healthy brain function and processing, which is displayed in the reduced cognitive scores. Interestingly, *BDNF* exon I methylation increased with age, albeit the association was weak, and so to some extent could be contributing to the cognitive ageing found within the population. To our knowledge, this is a novel observation of a background mechanism to cognitive ageing, although this is a tentative speculation that would require further research. However, to some extent this could bridge a gap seen within the analysis of *BDNF* methylation and cognitive ageing in which: 1) *BDNF* methylation has been associated with learning and memory function (Lubin et al, 2008; Roth et al, 2009), 2) *BDNF* methylation is a predictor of AD risk (Chang et al, 2014; Xie et al, 2017), 3) BDNF expression is associated with age-related decline in cognitive function (Komulainen et al, 2008; Erickson et al, 2010). Our identification of an age-associated reduction in *BDNF* methylation that is linked with cognitive decline, could potentially link this information together, while we further suggest that increased *BDNF* methylation could lead from healthy cognitive ageing into the progression of neurodegeneration.

4.6 Relationship between physical activity and *BDNF* regulation in the old adults

With DNA methylation and other epigenetic modifications providing some of the explanation for how environmental factors impact our genes and cause subsequent phenotypic changes, we considered lifestyle factors that have previously been linked with cognitive function, and analysed their impact on *BDNF* methylation.

Exercise is one such intervention that has been observed to reverse some of the cognitive deficit associated with ageing (Weuve et al, 2004; Ruscheweyh et al, 2011; Blondell et al, 2014). Therefore, we initially tested the cognitive function of the old population, between the participants who were classified as physically active and those who were inactive. No

significant differences were found in any of the cognitive functioning scores between the active and inactive groups, which is opposed to what has previously been investigated (Table 3.11). A significant amount of research has shown exercise to produce a number of positive changes in neurological function, and the mechanism by which this is achieved is well categorised (Marosi et al, 2014), however it is not clear whether there is a minimum exercise requirement to induce these changes. Previous studies have used exercise interventions that had a similar level of exercise regime to what was required to be in the MyoAge physically active group (Erickson et al, 2011), and had shown improvements in memory. This suggests that the MyoAge population appear to be undergoing sufficient aerobic exercise required to induce neurological changes. Research from the same intervention experiment have also reported improvements in executive function mediated by changes in serum BDNF levels (Leckie et al, 2014), but interestingly observed that these improvements were most pronounced in participants over 71 years of age. This suggests that this mechanism of action may only be significant to participants who already have neurological changes within their brain, that is inhibiting its function, and which may be the precursor to further cognitive decline or neurodegeneration. The old adults in the MyoAge cohort are all community-dwelling and high performing regardless of their classification of physical exercise, and have displayed no signs of neurodegeneration.

It is possible that exercise within the old population of the MyoAge group could still be inducing the molecular pathways that have been shown to improve cognitive function, and that it still may be helping them maintain a certain level of neurological health that could in the future counteract further progression of neurological decline. As BDNF has been widely shown to be one of the key mediators of these interventions (Erickson et al, 2011; Ruscheweyh et al, 2011; Leckie et al, 2014; Marosi et al, 2014), we studied the association of plasma BDNF with activity levels in the old population. Contrary to the majority of information, there were no significant differences between the active and inactive populations. While this result was not expected, our previous findings with unexpected associations with plasma BDNF levels meant it was not surprising. Further analysis will be required to decide whether the plasma BDNF readings are true representations of the MyoAge cohort, and whether they present a true difference to

information already published. A possible consideration is that a lot of research is on exercise interventions that are observed over a relatively short period. Hopkins et al (2011) investigated the effect of voluntary exercise between groups of adolescent and adult rats. The exercise intervention showed increased BFNF levels and improved novel object recognition, which is in line with the other research, however, when the old rats were tested two weeks after the intervention, it was found that these changes had reverted back to their previous levels. Therefore, maintained levels of exercise over a long duration should be considered in further investigation of cognitive intervention.

The study took into consideration the observations of changes in BDNF levels from previous studies, as well as the fact that the exercise levels in our physically active group were well maintained across a long duration. From this, we decided that if underlying epigenetic mechanisms were contributing to the changes previously observed, then there might be changes in *BDNF* methylation between the old active and inactive populations in the MyoAge cohort. When this was investigated, it was found that *BDNF* exon IV exhibited more methylation in the inactive population, but perhaps strikingly, the old active population had lower *BDNF* exon IV methylation than even the young population (Table 3.12). This was partly explained when the old population was stratified by gender, as within the female population both the active and inactive groups had lower *BDNF* exon IV methylation scores than the young population (Table 3.13). This trend was not picked up in previous analyses as no significant difference was shown between young and old females. While the difference is statistically negligible, the reduced *BDNF* methylation with age is opposite to the raised methylation levels witnessed in this study, in *BDNF* exon I, and others (McKinney et al, 2015), which goes to show methylation is a dynamic process which does not interact equally across the genome. Even with this surprising observation, the old female population still exhibited reduced methylation levels in the active population than the inactive. In addition, we distinguished that the old female population also had reduced *BDNF* exon I methylation in the active population, although this was a weaker association. This suggests that there is a gender specific role for exercise to reduce the levels of methylation at the promoters of the *BDNF* gene. While it has not been observed within this study, this suggests *BDNF* methylation as a potential underlying mechanism for how exercise increases *BDNF* transcript expression (Oliff et al,

1998; Vaynman et al, 2004) and subsequent BDNF expression (Erickson et al, 2011; Ruscheweyh et al, 2011; Leckie et al, 2014; Marosi et al, 2014). Further investigation must be done to develop understanding of how epigenetic regulation mediates exercise-associated increases in BDNF levels, but this result has also been established in rat models. Gomez-Pinilla (2011) has found that exercise reduces methylation of *BDNF* promoter IV, like our findings, while further showing that exercise increased the levels of phosphorylated MeCP2, CREB and activated CaMKII, which are associated in the regulation of *BDNF* (Vaynman et al, 2003; Zhou et al, 2006). They also showed that exercise enhanced the binding of acetylated histone 3 at *BDNF* promoter IV, which again is associated with increased *BDNF* expression (Tsankova et al, 2006; Tian et al, 2010). Taken together, these findings present the epigenetic regulation of the *BDNF* gene as a potent mediator of exercise-association changes in BDNF expression, which might be attributable to improvements in cognitive function.

4.7 Relationship between education and *BDNF* regulation in the old adults

Where physical exercise is an environmental stimulus that can produce short-term epigenetic alterations that impact cognitive function, education is proposed to produce more long-term alterations to neurological function that can impact cognitive function years after the event has taken place. However, it has been observed that education is associated with higher cognitive functioning in older age only when neuropathology is absent or mild, whereas with the onset of neurodegeneration, education does not slow the rate of cognitive decline (Koepsell et al, 2008; Zahodne et al, 2011; Arenaza-Urquijo et al, 2017). A reason for this is that education is one of a group of factors which contribute to a brain and cognitive reserve (Stern, 2009; Stern, 2012; Franzmeier et al, 2017) that involve two key concepts. 1) Brain reserve is the passive model where increases in brain size, neuronal density and synaptic connections allow the brain to maintain more advanced neuropathology before symptoms are expressed and 2) Cognitive reserve is a more active model in which pre-existing cognitive processes are called upon as compensation to cope with the dysregulation of other pathways affected by neuropathology (Stern et al, 2002). To test this theory, we analysed the effect that prior education attainment had on current cognitive functioning in the old adults. It was observed that there was a significant trend in which the further a participant had

progressed in their education, the higher their cognitive functioning scores were (Table 3.14). This association was only observed within the female population however, while the male population did not show a significant trend across all the education groups, they did display a weaker improvement in cognitive functioning in those who had achieved a university education compared to those who had achieved a basic education (Table 3.15). This agrees with previous research that within a healthy ageing cohort, education contributes to these reserves and improves cognitive functioning in later life.

It has been previously observed that BDNF plays a significant role in developing cognitive reserves (Stern, 2012; Ward et al, 2015; Buchman et al, 2016). This appears to be due to BDNF's involvement in promoting synaptic plasticity and LTP (Lu et al, 2013; Bekinschtein et al, 2014), that are also important for BDNF's role in learning and memory (Egan et al, 2013; Cunha et al, 2010). When BDNF was analysed against measures of education in the MyoAge cohort, a weak trend was observed in the total population, that plasma BDNF levels were increased in the participants who had progressed with their education (Table 3.16). This appears to support the results previously acquired, but caution should be exercised as to how much of an effect plasma BDNF has over brain and cognitive reserves. Though as previously mentioned BDNF does actively cross the blood brain barrier (Poduslo et al, 1996; Pan et al, 1998).

While this study did display minor associations between plasma BDNF levels and educational attainment, there were three key lines of thought that prompted our analysis of *BDNF* methylation and education, which are related to our considerations for *BDNF* - methylation and cognition (Section 4.5). Firstly, *BDNF* methylation has been associated with learning and memory in a lifelong manner (Lubin et al, 2008), therefore any epigenetic changes during early life exposures such as education may be preserved to some extent for a long duration (Kundakovic et al, 2015). Secondly, BDNF expression is associated with protection against cognitive decline (Buchman et al, 2016), suggesting it has some association with cognitive reserve. Thirdly, *BDNF* promoter methylation has been suggested to be a risk factor for AD, meaning that *BDNF* methylation might be inhibiting the ability of BDNF to contribute to the brain and cognitive reserves, resulting in an increased incidence of AD. When we analysed *BDNF* methylation against educational attainment, we observed some weak reductions in *BDNF* exon IV methylation with the

further the participants had progressed with their education (Table 3.17). There was a significant reduction in *BDNF* exon IV methylation in participants who had attained a university education compared to those who had achieved a basic education. We believe that there are two mechanisms in which education-associated changes in *BDNF* methylation may predict cognitive function in later life. Exposure to education and similar neurological enrichment appears to reduce the amount of methylation on the promoters of the *BDNF* gene, allowing increased BDNF expression to aid memory consolidation (Lubin et al, 2008). This firstly increases neurogenesis, LTP and synaptic density within the brain (Lu et al, 2014), enriching the brain and cognitive reserve that is maintained across a lifetime (Stern, 2012). These reserves are displayed with higher cognitive capabilities in old age. Secondly, *BDNF* methylation appears to be maintained to some extent across a lifetime (Kundakovic et al, 2015; Unternaehrer et al, 2015), therefore early changes in methylation could underlie long-term changes in *BDNF* gene expression. Higher *BDNF* gene expression as a result might provide protection in later life against cognitive ageing and neuropathology (Buchman et al, 2016). This theory is admittedly wide spanning and partially speculative, with many limitations, including only a cross-sectional analysis of *BDNF* methylation, a still uncertain understanding of the relationship between peripheral methylation and biological function in the brain and relatively small methylation changes. However, we believe there is enough supporting research to warrant further investigation.

4.8 The role of the *BDNF* Val66Met genotype in cognition

Epigenetic stimuli are not the only means of regulating BDNF expression, as genetic factors such as common genetic variants have been established as influencing the biological pathway of BDNF. The SNP rs6265 (Val66Met) results in a valine to methionine substitution at codon 66 in the pro-domain of BDNF which directly affects the structure of pro-BDNF, inhibiting the activity-dependent secretion of BDNF (Chen et al, 2004). It has been widely implicated in affecting cognitive function (Egan et al, 2003; Hariri et al, 2003; Kambeitz et al, 2012; Ward et al, 2015) and brain morphology (Kambeitz et al, 2012), as well as moderating age-associated cognitive decline (Miyajima et al, 2008; Sublette et al, 2008; Erickson et al, 2008; Lim et al, 2013). Results from this study displayed a significant reduction in working memory capacity, as well as a weaker reduction in executive

functioning, in participants who were either Val/Met heterozygous or Met/Met homozygous, in the male population (Table 3.18). Specifically, the old male population had a significant reduction in working memory capacity in participants who were either Val/Met heterozygous or Met/Met homozygous (Table 3.20). This result supports the research that has found that *BDNF* Met carriers perform worse on tests of cognitive function and memory (Egan et al, 2003; Miyajima et al, 2007; Nagel et al, 2008; Lim et al, 2013). On the other hand, we did not find that Val/Met heterozygous or Met/Met homozygous perform worse on fluid intelligence and executive functioning (Sapkota et al, 2017). A number of studies have found that the Val allele is indicative of increased age-related cognitive decline (Erickson et al, 2008; Nagata et al, 2012), and a recent meta-analysis compounds the inconsistent findings presented in the literature (Mandelman and Grigorenko, 2012). There are various reasons for these discrepancies that should be considered in future investigations, including, the heterogeneity of Val66Met amongst different ethnic populations, age of the participants, association with other genotypes (Wollam et al, 2015) and the interaction of Val66Met with environmental stimuli across a lifespan.

We considered whether the association between the Val66Met polymorphism and cognitive functions found in the MyoAge cohort was attributable to changes in BDNF expression. When this was analysed, it was found that there were no significant changes in plasma BDNF levels between participants who were Val/Val homozygous and those who were either Val/Met heterozygous or Met/Met homozygous (Table 3.21). This appears to be in agreement with previous observations (Egan et al, 2003; Chen et al, 2006; Frielingsdorf, 2010), although there has been some suggestion that the Met allele is associated with reduced BDNF levels (Ozan et al, 2010). This may be due to the different secretion mechanisms of peripheral cells compared to neuronal cells, as the Met allele only has been shown to effect depolarization-dependent secretion (Egan et al, 2003). So, an effect of the *BDNF* Val66Met polymorphism on peripheral BDNF levels might not be replicated within the brain.

4.9 Relationship between *BDNF* gene expression in the prefrontal cortex and age

As previously described, BDNF has been observed to be influenced in an age-dependent manner (Erickson et al, 2010; Leckie et al, 2014). Notably, the majority of research into BDNF, including the work of this study in the MyoAge cohort, has been performed in the peripheral systems. This can provide great insight into the role of BDNF, however, since BDNF is most biologically active within the brain, we analysed the expression of *BDNF* from prefrontal cortex samples from the Dyne-Steele cohort, as described previously. Although, the Dyne-Steele population consists of one age group, there was still an age range of over 30 years, and therefore we considered whether there were late onset age-associated changes in *BDNF* gene expression across the old population. Since age is the greatest risk factor for the onset of AD (Alzheimer's Association, 2017), we controlled for neuropathology within the analysis. This study found that there was a negative association between age and *BDNF* exon IV expression, as well as a weaker negative association between *BDNF* total expression, both of which were not attributed to neuropathology onset (Table 3.22). It has previously been well described that *BDNF* expression declines with age, both in healthy ageing (Croll et al, 1998; McKinney et al, 2015; Oh et al, 2016) and ageing associated with neurodegeneration (Tapia-Arancibia et al, 2008; Zuccato and Cattaneo, 2009). While our results agree with the consensus, we have also shown that *BDNF* expression maintains a negative association with age within an old population (72-104 years old), regardless of neuropathology.

4.10 Relationship between *BDNF* gene expression in the prefrontal cortex and cognition

Whereas other studies have compared *BDNF* gene expression between young and old populations, this study has shown that *BDNF* expression continues to decline with advancing age. Since BDNF has been associated with age-related cognitive decline (Komulainen et al, 2008; Erickson et al, 2010), we hypothesised that continued reduction in *BDNF* gene expression in later life may be responsible for the continued or accelerated cognitive decline observed in old age (Laske et al, 2011; Forlenza et al, 2015). This study showed that there was a significant association between *BDNF* exon I expression and impaired performances in memory and speed tests, as well as with a faster rate of decline in memory (Table 3.24). This finding is unexpected as it suggests that increased brain

BDNF expression is contributing to poorer memory performances. Considering that it is still not established what role neuropathology has in altering the BDNF mechanism, and that dementia induces a faster rate of cognitive decline than in normal ageing (Yu et al, 2012), we concluded that controlling for neuropathology was not enough to account for variation in *BDNF* expression and cognitive decline between the healthy ageing and AD groups. To test whether there are differences in the BDNF mechanism between these ageing groups, we stratified the population between healthy ageing and AD, and reanalysed *BDNF* expression against cognitive function. In the neurologically healthy population, it was shown that *BDNF* exon IV expression was weakly associated with improved memory as well as a slower decline in memory. Strikingly, *BDNF* expression appears to have a different function in AD, as it was observed that *BDNF* exon I and total *BDNF* expression are detrimental to performances in memory tests, as well as being associated with a faster rate of decline of memory (Table 3.25). This suggests that the biological mechanism of BDNF is different between healthy and disease-associated neurological ageing, and might be altered to compensate for neurodegeneration. Due to the inaccessibility of the brain during longitudinal testing, there are limited results on the role of BDNF in the brain during ageing. Various studies have shown that peripheral BDNF levels are associated with improved cognitive function and slower decline (Komulainen et al, 2008; Laske et al, 2011), including our own. It is still uncertain how peripheral BDNF levels predict function with the brain, although it has been associated with an age-associated decline in hippocampal volume (Erickson et al, 2010). For the healthy ageing group, our findings appear to support observations previously made that increased BDNF expression (Hattiangady et al, 2005), and *BDNF* gene expression (Tapia-Arancibia et al, 2008; Oh et al, 2016) in the brain are associated with an improved outlook regarding age-associated cognitive decline. Conversely, our findings from the AD group vary from what has previously been observed. Buchman et al (2016) analysed the *BDNF* gene expression from a cohort of over 500 older adults who had underwent longitudinal cognitive testing, clinical testing for AD and post-mortem neuropathological testing. They found that higher *BDNF* expression is associated with reduced rates of cognitive decline, however this association was strongest in the participants with Dementia, whereas they found no significant association between *BDNF* expression and cognitive decline in participants with no cognitive impairment. This suggests that BDNF works as a compensatory

protective mechanism against the damage caused by neurodegeneration. While our results contrast with this, we hypothesised that the association of *BDNF* expression with faster rates of memory decline might also be due to compensation rather than causation (Diniz and Teixeira, 2011). Our reasoning behind this is that *BDNF* expression was predominately associated with worse memory results in the AD group, however apart from one association with a faster rate of decline in vocabulary, there were no other associations observed. The faster rates of memory decline are indicative of hippocampal and cortical atrophy (Dubois et al, 2014) associated with AD, therefore the affected and surrounding neurons might increase expression of *BDNF* in an attempt to induce neurogenesis to compensate for damage (Budni et al, 2015; Song et al, 2015). Burbach et al (2004) found that *BDNF* mRNA was being upregulated around the tissue surrounding plaques, as well as significant increases in cortical BDNF levels with increased β-amyloid load. There are still inconsistences across multiple investigations into the role of BDNF in AD (Song et al, 2015), suggesting that there are factors not being considered in this relationship. The differences in BDNF responses shown between our study and Buchman et al (2016) may be due to the multifactorial nature of AD. Accumulated neuropathology might simply overwhelm BDNF compensation, or there might be genetic differences between European and American populations that alter the response of BDNF in AD. Overall, there appears to be a growing consensus that BDNF is protective against both normal and AD neuronal ageing, and further research is essential to understand how BDNF interacts with all systems of the brain, including inflammation (Calabrese et al, 2014), in response.

4.11 Relationship between *BDNF* gene expression in the prefrontal cortex and neuropathology

To further our understanding of the reasons why *BDNF* expression was associated with impaired memory and faster decline in the AD group, we analysed the association between *BDNF* expression and neuropathology, in order to determine whether BDNF was responding to the accumulation of plaques and neurofibrillary tangles (Burbach et al, 2004). We determined that there were significant differences in total *BDNF* expression with increased β-amyloid load (Thal), as well as this there were significant differences in *BDNF* exon I with increased neurofibrillary tangles (Braak) (Table 3.26). Further post-hoc

analysis found that there were no significant difference between the individual groups. Nevertheless, they both showed interesting trends with initial accumulation of β-amyloid and neurofibrillary tangles inhibiting *BDNF* expression, only for *BDNF* expression to recover during intermediate neuropathology, however *BDNF* expression again falls with advanced neuropathology (Figure 3.14, results). While the trend is statistically weak, there is evidence to support it, such as changes in BDNF around the tissue surrounding plaques have been observed (Burbach et al, 2004). Zussy et al (2011; 2013) have previously observed that BDNF is significantly responsive to cerebral β-amyloid injection, significantly increasing in the prefrontal cortex and hippocampus 1 week after injection, and then decreasing in the ensuing weeks. This transient response has been shown to partially compensate for the damage to neurons caused by β-amyloid (Rantamäki, 2013), as well as inhibiting the generation of β-amyloid (Gralle et al 2009; Rohe et al 2009). As we have suggested, β-amyloid affects the function of BDNF within the brain (Meng et al, 2013; Zussy et al 2013). It appears to inhibit the PI3K-Akt-mTOR signalling pathway downstream of BDNF-TrKB binding (Jimenez et al, 2011), interfering with the support BDNF provides to neuronal plasticity and survival. Moreover, β-amyloid directly inhibits *BDNF* transcription by interfering with the activation of CREB, stopping it from binding to the *BDNF* promoters (Meng et al, 2013; Liu et al, 2013; Palomer et al, 2016). While BDNF is reactive to β-amyloid and actively works to combat it, β-amyloid also inhibits BDNF. Our results appear to support this balance, with initial β-amyloid accumulation overwhelmed by increased expression of *BDNF,* however further accumulation of β-amyloid tips the scales and *BDNF* expression is suppressed, leading to further acceleration of neurodegeneration. This supports our findings of altered *BDNF* expression between healthy ageing and AD, as in healthy ageing, any presence of neuropathology is countered by increased *BDNF* expression, therefore BDNF is not inhibited from its normal promotion of neurogenesis and synaptic plasticity. In AD, neuropathology has progressed to a point where it is overwhelming the compensatory mechanism of BDNF, while *BDNF* continues to be upregulated in a crisis response to the accumulated damage, although its function is being inhibited. While, this might improve the understanding of the differences between age-associated cognitive decline and AD, it is still a microcosm of the neurological condition. Therefore, research should progress to investigate this relationship.

4.12 Relationship between *BDNF* methylation and *BDNF* gene expression in the prefrontal cortex

Since we have observed a mechanism for *BDNF* methylation to mediate the decline in BDNF in the plasma, and that it was attributable to age-associated cognitive decline in the MyoAge cohort. We tested the same principle in the prefrontal cortex to determine if methylation is associated with *BDNF* expression in the brain. As expected *BDNF* methylation was lower in the prefrontal cortex (Dyne-Steele) than the blood (MyoAge), indicating higher levels of *BDNF* expression in the brain. It was observed that there was a weak negative association with *BDNF* exon I methylation and expression (Table 3.27). Notably this association was only preserved, albeit weakly, in the healthy ageing group, while no significant association was found in the AD group (Table 3.28). This suggests that the normal epigenetic regulation of *BDNF* is deregulated or bypassed in AD. Various neurological disorders have been shown to affect *BDNF* methylation (Roth et al, 2009; 2011; Autry and Monteggia, 2012), showing that *BDNF* methylation is susceptible to adverse environments. Whereas the effect of AD on *BDNF* methylation remain inconsistent, with both findings of higher (Chang et al, 2014; Nagata et al, 2015) and unchanging (Bakulski et al, 2012; Carboni et al, 2015) methylation levels in AD. A negative association between *BDNF* methylation and *BDNF* expression in an older group has been reported previously (Keleshian et al, 2013), but no previous reports of differences in *BDNF* methylation between control and AD participants was found. Mastroeni et al (2010) found that various epigenetic markers and regulators, including DNMT1 and the MeCP1/MBD2 methylation complex, were significantly decreased in AD, suggesting that AD does directly interfere with epigenetic regulation. While only observed in one of the *BDNF* transcripts, the change in association between *BDNF* methylation and expression from healthy ageing to AD, may indicate that the *BDNF* epigenetic regulation is compromised in AD. From another perspective, AD may induce transcriptional and posttranscriptional alterations that bypass the epigenetic regulation, such as how β-amyloid inhibits CREB activation and subsequent *BDNF* activation (Meng et al, 2013; Liu et al, 2013). Caution should be exercised with this observation until further investigation has taken place, as the low numbers may inhibit a significant observation being observed in the AD group.

4.13 Relationship between *BDNF* methylation in the prefrontal cortex and cognition

BDNF exon I methylation was observed to be weakly associated with worse baseline memory scores and a faster rate of decline in memory (Table 3.29). As *BDNF* methylation at the promoter sites inhibits transcription of *BDNF*, this supports the hypothesis that higher *BDNF* methylation would limit the expression of BDNF, and its neurotrophic properties. While the two populations are separate, this finding supports our observation from the MyoAge cohort. The differences between *BDNF* methylation in AD and healthy ageing remain inconclusive, as described above, but the current body of literature appears to show *BDNF* methylation as a risk factor for further cognitive decline and progression to AD (Xie et al, 2017). We observed above that AD neuropathology might also influence the relationship between *BDNF* methylation and expression and therefore we attempted to determine whether this was mirrored in the association with memory. However, no significant associations were observed in either group (Table 3.30).

4.14 Regulation of H3K27 acetylation at *BDNF* exon IV in the prefrontal cortex

There are additional epigenetic regulatory mechanisms aside from DNA methylation such as chromatin and histone marks. Therefore, considering the lack of associations between *BDNF* exon IV methylation and *BDNF* expression in the brain, and the relatively low levels of methylation at this locus in the brain (Table 2.2, Methods), another epigenetic marker, histone H3k27 modification, was considered as a potential mediator of *BDNF* exon IV expression. This marker is usually associated with active gene transcription. (Palomer et al, 2016). In addition, we considered the possible role of polymorphisms in regulating epigenetic control of *BDNF*, i.e. the genetic-epigenetic interaction that may be involved in *BDNF* expression. Our choice of rs56133711 was based on its high frequency in the European population and it was the closest functional polymorphism to *BDNF* exon IV and H3K27Ac binding sites. After we found that H3K27Ac was binding to the *BDNF* exon IV promoter region, we performed allelic specific ChIP pulldown, in which we found that the H3K27Ac marker associated more with the A allele of rs56133711 (Figure 3.21). This suggests that the polymorphism does affect H3K27Ac binding. As H3K27Ac is associated with active gene transcription (Palomer et al, 2016), we next considered whether this allele specific association contributed to *BDNF* expression. However, we found no

association between polymorphism and gene expression (Table 3.31). Although no statistical significance was found, this points to the multiple regulation mechanisms that need to be considered within *BDNF* expression. Most likely activity dependent promoters such as *BDNF* exon IV require more complex regulation than simply associating with either methylation, histone acetylation or other factors individually.

4.15 Limitations

Several important limitations of the study need to be mentioned. With the MyoAge cohort, while taking peripheral measurements allowed for DNA and protein samples to be taken at a close timepoint to cognitive testing, the functionality of peripheral BDNF as a biomarker for cognitive decline remains inconclusive. Also, cross-sectional cognitive testing limited the studies ability to draw conclusions relating to cognitive decline.

The Dyne-Steele cohort had relatively low numbers in comparison to some other studies observing BDNF in the brain, reducing the power of our findings, especially when we stratified the population. The fact that different time points were used for cognitive testing and molecular testing of the prefrontal cortex should be considered when observing any associations between BDNF and cognition. Furthermore, it is inconclusive how much of an effect small changes in methylation, even around promoters, has on transcription.

4.16 Summary

Even with these considerations, there are many implications that can be drawn from this study. We have contributed further to the hypothesis that *BDNF* methylation contributes to cognitive ageing, and could potentially lead to further risk of AD. Factors such as exercise and education can affect *BDNF* methylation throughout life, which may contribute to the varying presentations of cognitive ageing and AD. Moreover, AD inhibits the function of BDNF and interferes with the epigenetic regulation of *BDNF*. *BDNF* methylation inhibits *BDNF* expression and cognitive function in older age, but these observations are weaker in AD. We have also found a novel genetic-epigenetic interaction, which could further contribute to the larger picture of *BDNF* epigenetics and cognitive ageing.

These findings highlight the potential for further understanding of *BDNF* regulation, with implications for both cognitive ageing, and the development and progression of AD. As described, further investigation should be performed into the individual lines of research, but if they support the findings of this study, then BDNF should be considered as part of an underlying biomarker framework for the neurological changes that could prelude AD.

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