Epigenetics in mental and neurodegenerative disorders

Citation for published version (APA):

Iatrou, A. (2018). *Epigenetics in mental and neurodegenerative disorders: The unusual suspects*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.20180712ai>

Document status and date:

Published: 01/01/2018

DOI:

[10.26481/dis.20180712ai](https://doi.org/10.26481/dis.20180712ai)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

COPYRIGHT @ Artemis Iatrou, Maastricht 2018
GRAPHIC DESIGN: Andreas Gugliotta
LAYOUT DESIGN: Yorgos Garofalakis
PUBLISHED BY: IPSKAMP PRINTING
ISBN: 978-94-028-1099-8
PRINTED BY: IPSKAMP PRINTING

EPIGENETICS IN MENTAL AND NEURODEGENERATIVE DISORDERS; THE UNUSUAL SUSPECTS

DISSERTATION

to obtain the degree of Doctor at Maastricht University, on the
authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans, to be
defended in public on Thursday 12th of July 2018, at 10:00 hours

by
Artemis Iatrou

PROMOTOR

Prof. dr. Bart P.F. Rutten

CO-PROMOTORS

Dr. Daniël L.A. van den Hove

Dr. Gunter Kenis

ASSESSMENT COMMITTEE

Prof. dr. Jos C.S. Kleinjans

(Chair)

Prof. dr. Therese A.M.J. van Amelsvoort

Prof. dr. Arfan Ikram

(Erasmus MC, University Medical Center Rotterdam)

Dr. Lars M.T. Eijseen

Dr. Rebecca G. Smith

(University of Exeter & Kings College London, UK)

To Yiannis.

01.

General introduction

page 6

02.

Epigenetic dysregulation of brainstem nuclei in the pathogenesis of Alzheimer's disease: looking in the correct place at the right time?

page 36

03.

Methylomic and hydroxymethylomic profiling of the dorsal raphe nucleus in Alzheimer's disease

page 74

04.

Epigenetic variation of the miR-137 gene in Alzheimer's disease

page 108

05.

On the epigenetic theory of major psychosis: schizophrenia- and bipolar disorder- associated methylomic signatures in the cerebellum

page 132

06.

Memory restoring properties of a synthetic TrkB agonist

page 172

07.

General conclusion

page 194

08.

Summary

page 205

09.

Valorization

page 211

10.

Curriculum Vitae

page 216

11.

List of publications

page 218

12.

Acknowledgments

page 220

S
T
N
E
T
N
O
C

1 General introduction

Epigenetics with a focus on DNA methylation	p.8
Alzheimer's Disease	p.10
Schizophrenia	p.14
Bipolar Disorder	p.16
The role of epigenetics in neurodegenerative and neurodevelopmental disorders	p.18
Overview of thesis	p.19
References	p.22

Mental and neurological disorders of degenerative and developmental nature, particularly Alzheimer's disease (AD), schizophrenia (SZ) and bipolar disorder (BP), are nowadays among the most challenging health problems as they remain practically incurable due to lack of effective treatment strategies. The need for efficient therapeutic and diagnostic options is more urgent than ever now that the aging population is all the more increasing and that socio-political factors cause higher discrimination between social strata as well as among individuals with diverse ethnic backgrounds.

The pathological profile and symptomatology of each of these disorders is rather unique, but collectively they are among the leading causes of cognitive, emotional and social disability in younger and older populations. The prevalence of these disorders in numbers are 4% of the aging population for AD and 1% of the population worldwide for any of the two psychiatric disorders, SZ or BP [1, 2]. The noted dysfunction in these domains significantly reduces the quality of life, depriving the afflicted individuals from a normal life with adequate social support from their families and loved ones. Additionally, the universal research effort devoted into discovering effective treatment has yet to yield encouraging outcomes, resulting in a relentless increase in economic and human costs.

The common denominator of ineffective treatment and delayed diagnostic options is the inconclusive knowledge on the aetiology of the disease. Genetic studies have identified genes responsible for the Mendelian inheritance pattern and have shed ample light in the basic underlying biological processes. Many of these genes have also been validated via meta-analyses and additionally polygenic scores have been developed for each disease in order to predict case/control status [2-4]. However, genome-wide studies (GWAS) have indicated that in all these diseases, the common generic variants account for a very small percentage of the attributable risk, while the rare ones have larger effects. On top of that, while subtypes of these diseases are highly heritable, observations of discordant disease outcomes from concordant twins come to establish the notion of "heritability gap".

Cumulative evidence has indicated that environmental influences throughout the lifespan and in particular during development are linked to disease phenotypes. The breadth of the definition of “environmental influences” includes infectious agents, pathogens and pollutants, illicit compounds, traumatic brain injuries as well as nutritional deficiencies but also physiological, psychological and social stressors, such as maternal stress during pregnancy, low birth weight, childhood trauma, socioeconomic injustice and status, etc. Environmental risk factors *in toto* or individually influence susceptibility to mental illnesses. However, their role is considered contributory rather than causative, since exposure to them does not always infer deviant, disease-related pathology. It has been suggested that a defective genetic make-up could induce differential interindividual vulnerability to environmental stressors, or vice versa, an environmental risk factor could have different effect in individuals with different genotypes, both scenarios leading to disease manifestation. This notion is now a tenet of modern science, referred to as “gene x environment interactions”. Therefore, the aetiology of a disease seems to lie beyond genetic predisposition, and/or environmental influences *per se* and additional mechanisms are operative.

Epigenetic modifications have been proposed as one of the mechanisms filling in the “heritability gap” and mediating “gene x environment interactions”. To date, knowledge collected from epigenetic research including epigenome-wide association studies (EWAS) complements the established genetic and polygenic approaches, and offers new hope for elucidating the complex biological mechanisms underlying disease aetiology and pathology. In the following chapters, an introduction to epigenetics is provided as well as their involvement in AD, SZ and BP.

Epigenetics with a focus on DNA methylation

While the term epigenetics was originally coined by Conrad Hal Waddington in 1942 [5] in order to define the study of causal mechanisms of development mediating the expression of specific cellular phenotypes, it was only used with its current meaning as from 1996. Riggs and Russo then defined epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” [6]. This definition has been complemented since then, currently including notions of reversibility of these changes [7] and transient mechanisms that are involved in the initiation of the new epigenetic state that are different from the ones required to maintain them [8] (see [9]). Extending beyond normal cellular development and differentiation, epigenetic modifications can transiently imprint environmental effects on gene expression throughout the life span. The umbrella term, epigenetic modifications, e.g. covers DNA methylation and hydroxymethylation, chromatin remodelling and non-coding RNA (ncRNA) regulation. And while each one of them has

a different function, they all are highly interdependent, with for example DNA methylation working in harmony with histone modifications and microRNAs (miRNA) to regulate transcription and to ensure the maintenance of transcriptomic homeostasis in the brain.

Epigenetic DNA modifications conventionally refer to the addition of a chemical group to cytosines (C) throughout the genome, hence establishing five distinct cytosine states: unmodified cytosine (C), 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Figure 1A). DNA methylation, the most studied epigenetic modification, commonly refers to the addition of a methyl group to an unmodified C at the 5' position on the pyrimidine ring, forming 5mC, by DNA methyltransferases (DNMTs). Methylation is catalysed and initially established by the *de novo* DNMT3a and DNMT3b during early embryogenesis and it is maintained by DNMT1, which acts by recognizing and copying methylation patterns during cellular division. While this modification occurs with higher frequency at cytosine-phosphate-guanine (CpG) islands across the genome, non-CpG methylation has recently been confirmed [10, 11]. The effect of DNA methylation on gene transcription is highly dependent on its location in or around the genetic locus [12]. Generally, high levels of DNA methylation in CpG islands located within the 5' gene promoter are associated with disruption of the transcriptional machinery and, thus, gene silencing [13]. This is achieved via the disruption of the binding of transcription factors [13] and the attraction of methyl-binding proteins (MBP) that subsequently activate the histone tail modifying machinery eventually initiating chromatin compaction [11]. In contrast, gene body methylation is generally implicated in active gene expression [14-18], while intragenic DNA methylation has been shown to modulate alternative splicing [19-22]. The exact mechanisms mediating these differential effects remain to be elucidated.

While research in the realm of DNA methylation was initially approached with traditional biochemical approaches, recent scientific breakthroughs in epigenomic technology have boosted the field of neuroepigenetic studies. The advent of the Illumina microarray technology decisively changed the status of epigenetic research by providing cost-effective, high throughput methods for interrogating the genome systematically at a single base resolution to the scientific enterprise. The first HumanMethylation27 (27k array) BeadChip which interrogated < 27k CpG sites, was replaced in 2013 by the HumanMethylation450 (450K) BeadChip, examining more than 450k sites, which in turn is currently succeeded by the MethylationEPIC BeadChip, covering more than 850k CpG sites genome-wide. All these platforms follow the same rationale; they quantify the methylated (M) and unmethylated (U) signal intensities of a single cytosine and they calculate a customary index of DNA methylation fraction at this specific CpG site (β value) [3]. The input for the BeadChip can be either bisulfite (BS)- or oxidative (oxBS)-

converted DNA. Briefly, sodium BS treatment of denaturated DNA leads to deamination of unmethylated cytosine to uracil, which subsequently is amplified as thiamine via PCR reaction. On the contrary, 5mC and 5hmC get amplified as cytosines. In oxBS treatment, with the addition of an extra step, 5hmC is oxidised to 5fC before BS conversion, allowing for direct measurement of a specific 5mC signal as well as providing an indirect method for measuring 5hmC (Figure 1B).

Functional roles of DNA methylation within the CNS include brain development and neurogenesis, as well as the regulation of synaptic plasticity and long-term potentiation. Distinct DNA methylation signatures were reported in different brain regions, an observation which led to the hypothesis of methylation-regulated region-specific functional specialization of brain structures [34]. Further, this fact indicates that extensive de novo DNA methylation occurs during neural maturation and that these modifications are potential determinants of cell fate. With respect to neurogenesis, epigenetic factors have been accredited for maintenance of neural stem cell renewal as well as the proliferation and differentiation of new mature neurons [35]. Additionally, DNMTs are required for synaptic plasticity and facilitation of learning and memory via the induction of long term potentiation within the hippocampus [36]. And while hydroxymethylation was originally considered a mere intermediate product of DNA demethylation, vital physiological roles have been attributed to this modification in the meantime. Nowadays, 5hmC is associated with neuronal proliferation and maturation as well as synaptogenesis, and, more generally, with learning and memory processes [37-39].

Undoubtedly, the functional gamut of methylation and hydroxymethylation marks regulates crucial biological mechanisms in the CNS. Therefore, an epigenetic disequilibrium could influence brain homeostasis and trigger pathological cascades that lead to neurodevelopmental and neurodegenerative disorders. Moreover, while genetic and environmental factors have been repeatedly reported to contribute to the underlying cause of such disorders, notions holding aberrant DNA methylation (co-)responsible for the aetiology and progression of these pathological conditions have garnered much attention recently. To this end, the underpinning pathological events linked to neuropsychiatric disorders such as AD, SZ and BP have been associated with dysregulated epigenetic machinery.

Alzheimer's Disease

AD is a chronic, neurodegenerative disorder and the main cause of dementia. By now, its ever-increasing prevalence in combination with the lack of effective therapeutic solutions makes AD one of the most burdensome diseases for society, the economy and, at a more personal level, patients' families and relatives. While the clinical manifestation of the disease is discernible at approximately the 6th decade of the lifespan, with cognitive impairment, memory loss and day-

to-day debilitation, it is suggested that the incipient stages may start, yet remain asymptomatic, in young adulthood.

The main hallmarks of AD remain the extracellular amyloid beta ($A\beta$) plaques, which form degradation-resistant aggregates, and hyperphosphorylated tau protein that leads to the formation of intraneuronal neurofibrillary tangles (NFTs). Theories on the pathogenesis of the disease support that these two characteristic hallmarks lead to synaptic dysfunction and eventually neuronal cell loss, causing dramatic cortical and subcortical atrophy. Along with these hypotheses, many more have been added to the aetiological spectrum of the disease, namely energy metabolism, inflammation, and cell cycle control dysregulation, elucidating part of the pathophysiology underlying AD, but at the same time arresting development towards a unifying hypothesis for the pathogenesis of AD.

Human AD pathology is majorly detained within the CNS [40, 41]. There, the pathology propagates in a rather selective spatial and temporal manner with some regions being highly vulnerable to the aforementioned hallmarks at specific time points and others relatively resilient [42]. It is remarkable that the pathology particularly targets very specific neuronal types, which share long, late-myelinating and weakly myelinating axons [42, 43]. Thus, the initial manifestation of abnormal tau material is observed in the brainstem, and, more specifically, in the magnocellular nuclei of the basal forebrain, the oral nuclei of the brainstem raphe nuclei and the locus coeruleus (LC) [42-69]. From there, one can follow the propagation of the pathology to highly vulnerable subcortical areas and, later on, to high-order association areas of the neocortex [42, 49, 70]. Once cortical areas are damaged, the curtailment of intellectual functions begins, gradually leading to deterioration or even loss of executive functions, annotating the clinical phase of AD. Based on the observed tau progression, Braak and Braak introduced a 7-point system for staging AD representing the spreading pattern of tau pathology [51].

In AD, individual disease risk is determined by genetic, environmental and demographic factors, as well as interactions between them. From a genetic point of view, AD can be classified in two categories; the familial (fAD) and the sporadic (sAD) one. While the symptomatology and the progression of both forms are comparable, the aetiology is fundamentally different [71]. fAD accounts for only 1-5% of the disease cases and the existence of genetic mutations in specific genes is able to explain the majority of them. Mutations in the amyloid precursor protein (APP) gene and the presenilin (PS) genes PS1 and PS2 have been associated with fAD [72-79]. While APP is an $A\beta$ precursor, PS1 and PS2 represent γ -secretase-associated proteins, responsible for the cleavage of APP. Thus, loss-of-function detected mutations as seen in fAD promote $A\beta_{1-42}$ production, i.e. the $A\beta$ species that is more prone to aggregate [80].

Sporadic AD, the most prevalent form of AD, usually has a late-onset (> 65 years) and bares non-Mendelian traits. Most of the genes and genetic variants that emerged from GWAS have been robustly associated with AD, but only account for a fraction of susceptibility risk [[81]; for specific GWAS results see [82-85]]. Moreover, the mechanisms by which they influence the development and progression of AD remain to be elucidated. Indeed, even for the genetic risk factor with the highest population-attributable risk for sAD, the ϵ 4 allele of the Apolipoprotein E (APOE) gene, the mechanism behind its action has not yet been discovered [86]. Mounting evidence suggests that the increased levels of brain APOE ϵ 4 messenger RNA (mRNA) in AD patients are associated with dysfunctional clearance and aggregation of A β via various speculated, yet not proven, mechanisms affecting cerebral energy metabolism, neuroinflammation, neurovascular function, neurogenesis and synaptic plasticity [87]. In contrast to the approximately 12-fold increase in sAD occurrence in two ϵ 4 allele carriers, differential effects of other APOE isoforms have been shown, with the APOE ϵ 2 allele conferring protection against sAD, increased longevity and improved cognitive health [88].

Notwithstanding the highlighted connection between both familial and sporadic forms of AD with A β misprocessing, dysfunctional tau has also been suggested as the proximate cause of neurotoxicity in AD. Tau is a cytoskeletal protein largely associated with the stabilization of axonal microtubules in the CNS [89, 90]. In AD, hyperphosphorylated tau aggregates at the somatodendritic compartment of neurons forming paired helical filaments (PHF), which mainly constitute the NFTs [91-93]. NFTs are held responsible for defective neuronal function by means of microtubule dissociation and, consecutively, cytoskeletal deterioration, neuronal dysfunction and, ultimately, cell death. Tau misprocessing was for a long time considered a ubiquitous condition associated with ageing, since a similar pathological process is observed in other tauopathies [94]. Nevertheless, recent evidence has proven the opposite, i.e. that specific pathologic tau inclusions are AD-related. Tau derived from NFTs from AD patients can sequester normal tau and other microtubule-associated proteins (MAPs) and, furthermore, can cause the formation of new tau inclusions that are not only remarkably stable, but also produce single cell strain as opposed to other tauopathies [95]. Additionally, AD is not characterized by any mutations in the MAP tau (MAPT) gene, a common case in other general tauopathies [96, 97].

Despite the strong association between hallmark appearance and the incidence of AD, many individuals may carry the most salient risk factors for AD, as well as express profuse A β and tau pathology, but yet never clinically develop the disorder [76, 98-100]. Interestingly, even monozygotic twins can have discordant AD outcomes [99]. Such phenomenon can only be explained by a crucial dysregulation of the epigenetic machinery. Thus, in-depth knowledge on epigenetic modifications in AD, particularly DNA methylation, is ever increasing, elucidating the complex architecture of the disease.

In order to reveal the involvement of DNA modifications in the pathogenesis and physiology of AD, three different research strategies have been employed. Firstly, studies have examined global DNA methylation and hydroxymethylation levels in post-mortem brain tissue. Some of these studies showed a global decrease in DNA modifications within the hippocampus, temporal neocortex and entorhinal cortex [99, 101-103], while others reported elevated levels in the middle temporal and frontal gyrus [104]. The most recent one provided evidence for early stage modifications, but no global changes in methylation or hydroxymethylation in the entorhinal cortex during the later stages of the disease [105]. These reported results might have been highly inconclusive, but they mainly highlighted a potential role for the epigenetic machinery in AD and, furthermore, the tissue specificity of epigenetic modifications. Secondly, the (hydroxy)methylation profile of targeted genes, associated with disease pathology has been examined. The genetic locus of APP has been the focus of many research lines; yet, the outcomes are highly inconsistent with reported hypomethylation [106], hypermethylation [107] or no AD-related methylomic changes [108, 109] in the gene promoter. Beyond APP methylation profiles, a wide range of other candidate genes have been studied and differential methylation patterns have been detected in a number of them, namely PP2AC, S100A2, SORBS3, BDNF, SYP, NF- κ B, COX-2, TREM2 and MAPT (reviewed by [23, 76]). Thirdly, the advent of high-throughput methodologies led to the era of EWAS in AD. The first ever EWAS utilized the Illumina Infinium Methylation 27K array and identified 948 CpG frontal cortex positions harbouring 918 unique genes associated with late-onset AD, with the most significant probe residing in the TMEM59 gene [110]. Use of the same technology revealed four different loci annotating for DUSP22, CLDN15 and QSCN [111]. Upgrading towards the currently most widely used method for EWAS, the Illumina Infinium Methylation 450K array, the literature has been complemented with genetic loci that have been validated by independent studies [112-114]. The work presented in this thesis is the first EWAS to ever utilize the state-of-the-art technology of EPIC array and thoroughly distinguish and profile the methylome and hydroxymethylome, as well as outline the state of the unmodified cytosines in AD.

Schizophrenia

SZ is a diagnostic category of the psychosis spectrum disorders. The traditional diagnostic category of SZ comprises a combination of symptoms and has generally been conceived as a chronic neurodevelopmental disorder affecting approximately 1% of the population worldwide. The nature of some of the symptoms (e.g. paranoia, bizarre delusion, and disturbed reality testing) and the early onset of the disorder, in combination with its medication-refractory character in a proportion of patients and the stigma in society highly debilitates the patient's lifestyle and creates an enormous burden for the affected people and their relatives. The symptoms of this disease spectrum highly vary, but, overall, the disability stems from negative symptoms, portrayed by the lack or loss of e.g. a normal emotional range, social activities, and motivation, positives symptoms, which include executive dysfunction, hallucinations and delusions as well as cognitive impairment [115]. The relapse of positive symptoms in conjunction with numerous health comorbidities and concomitant suicide ideation usually result in a decreased lifespan by approximately 15 years [116]. Importantly, the social stigma associated with SZ undermines the public opinion concerning disease manifestation and weaves it with a hopeless chronic brain disease as well as notions of "split personality", most of the times resulting in extreme social isolation of the afflicted. To this end, it has been recently suggested that the term "schizophrenia" should be replaced with "psychosis spectrum syndrome" [117].

The main suspect for the pathophysiological abnormalities is aberrant neurotransmission, mainly involving the dopaminergic, serotonergic and glutamatergic systems [116]. Dopaminergic dysregulation is more closely associated with the symptomatology. Briefly, low nigrostriatal dopamine levels are linked to motor symptoms, and low mesocortical levels are more closely connected to negative and cognitive deficits. Increased mesolimbic dopamine levels on the other hand play a role on the positive symptoms. The involvement of the other two systems, i.e. the serotonergic and glutamatergic neurotransmitter systems, was discovered by the schizophrenia-like symptoms that LSD and ketamine/phenylcyclidine, respectively, were shown to induce. To this end, pharmacological compounds antagonizing both dopaminergic and serotonergic receptors were more effective in mitigating a wider range of symptoms. Additionally, dampening of SZ-associated NMDA receptor activity can alleviate from the negative and cognitive symptoms.

The aetiology of SZ, despite the body of research dedicated to it, still remains

highly elusive. The multifaceted character of the various endophenotypes encompassed in the spectrum allow for multiple underlying aetiologies, including genetic susceptibility and environmental influences. The high heritability rates suggest a genetic underlying cause, though GWAS have highlighted common genetic variants with small effects and rare genetic variants with higher yet not aggregated effects of risk for developing SZ. Furthermore, environmental and social factors have been reported to contribute to the development of SZ to individuals susceptible to the disease. Such factors are childhood trauma, minority ethnicity, urbanicity and lifestyle stressors such as drug abuse, social isolation as well as discrimination [115]. And while the naïve eye would assume that the genetic basis and the environmental stressors account for the aetiology of the disorder, twin studies bring epigenetics in the spotlight. The concordance rate between monozygotic twins for schizophrenia ranges from 40-65%, and empirical evidence cannot clearly credit non-shared environmental stressors as the factors explaining the remaining variation [118]. To this end, epigenetic involvement in the aetiopathogenesis of SZ is gaining ground, as epigenetic modifications could be both precipitants of the illness and susceptibility modifiers by bridging gene x environment interactions [119].

Studies using candidate-based approaches have identified differential methylation patterns in the vicinity of *COMT*, *RELN*, *DRD2* and *SOX10*, although replication studies failed to demonstrate similar patterns in *COMT* and *RELN* [120-123]. The first ever EWAS performed on the frontal cortex of SZ patients used a CpG island microarray and thus interrogated approximately 12,000 sites across the genome and illustrated disorder-specific methylation modifications in genes involved in neuronal development as well as glutamatergic and GABAergic neurotransmitter systems [124]. Two additional studies, utilizing the 450k array, focused on examining SZ-associated methylomic variation across brain regions (frontal cortex, hippocampus, striatum and cerebellum). The first study identified prefrontal cortex-specific loci that were significantly differentially methylated in SZ (*GSDMD*, *RASA3*, *HTR5A*, *PPFIA1*) and, most importantly, they highlighted that clusters of co-methylated genes associated with the diagnosis of schizophrenia were enriched for neurodevelopmental pathways [125]. In the second study, while no global methylation changes were detected, specific probes with significantly differential methylation exhibited consistent patterns across the brain regions under examination [126]. Four of these probes, *GSDMD*, *RASA3*, *PPFIA1* and *MYT1L* were common with the first study. Finally, the only study exploring the role of DNA hydroxymethylation in schizophrenia was performed in blood by using 5hmC- and 5mC-specific dot blots, where no significant changes were detected [127]. The work later presented in this book constitutes the first EWAS in the field of schizophrenia utilizing the EPIC array.

Bipolar Disorder

BP is a life-long disorder affecting approximately 1% of the population without discriminating among sex, ethnic origin, socio-economic status and similar factors that have been reported to affect the differential prevalence of other mood disorders. The initial manifestation of the symptomatology dates in early adulthood and is characterized by polarity between mania and hypomania with concurrent depression. The frequency and severity of mood changes has led to the classification of two types of BP; BP-I and BP-II, with the former involving periods of severe mood swings from mania to depression, while the latter is characterized by milder mood elevation, from episodes of hypomania to severe depression [128]. To this end, afflicted patients of both types present functional and cognitive deficits, and particularly BP-II patients often face suicide ideations as well as other psychiatric comorbidities, which significantly decrease their quality of life.

Early research lines were treating BP as a mood disorder solely, attempting to unveil underlying monoaminergic disequilibrium as the main suspect of the pathophysiology. Nevertheless, such dysregulation was not profoundly supported by empirical data. Mostly reduced neuronal resilience due to altered synaptic and neural plasticity has been observed in brain tissue from BP patients. This phenomenon has been mainly attributed to decreased expression of brain-derived neurotrophic factor (BDNF) and dysregulated neurotrophic and downstream pathways [129]. On the same time, other pathways affecting neuronal plasticity and connectivity subsume mitochondrial dysfunction, neuroinflammation and oxidative stress [130].

All the more insight is provided in the pathology and underlying molecular and cellular mechanisms of BP, yet its aetiopathogenesis remains elusive. According to some reports, BP is among the most heritable psychiatric disorders, but oddly enough the “heritability gap”, particularly between concordant twins remains a puzzling factor [131]. GWAS have reported and replicated many risk alleles that have a small effect, and interestingly they do overlap with SZ (i.e. *CACNA1C*, *TENM4*, and *NCAN*), contributing to the establishment of the polygenic risk score for BP [132]. The model most optimally explaining the variation in heritability in this disorder is a multifactorial one interlacing genetic and environmental factors [133]. Differential propensity of various environmental stressors in combination with genetic variants to disease manifestation have been reported in literature, including factors such as childhood trauma, stressful life events, lack of social support, obstetric complications, (maternal) smoking as well as *Toxoplasma gondii* exposure ([134]; reviewed by [135]).

As for AD and SZ, a composite factor able to explain both the “heritability gap” and the imprint of environmental stressors on the genome in light of BP is epigenetic mechanisms, and empirical evidence has already indicated that histone and DNA methylation changes are profound in BP patients. Focusing on DNA modifications, targeted approaches interrogating the profile of “usual suspects” have identified differential methylation patterns in proximity of the *BDNF* genetic locus [136-140], serotonin transporters genes [141-143] as well as SZ-associated genes, namely *RELN* [144, 145] and *MB-COMT* [146]. Global methylation studies main not have shown consistent results, still they highlighted the region-specific nature of methylation modifications in BP but also how prone they are to the effect of environmental stimuli such as insulin resistance, second-generation antipsychotics use and smoking [147].

Extensively analysed are the epigenome-wide modifications, as the conducted studies have profiled peripheral blood tissue [148-153] and *post mortem* one, i.e. (pre)frontal cortex [154-156], anterior cingulate gyrus [156] and cerebellum [157]. The majority of the studies have been performed in blood where various differentially methylated positions have been identified, elucidating further the pathophysiology of the disease as well as paving the way towards disease biomarkers. Recently one of these studies showed that BP patients exhibit accelerated epigenetic aging, in comparison to control, a phenomenon partially associated with aberrant mitochondrial DNA [153]. The “epigenetic clock” normally can predict the chronological age of the individual, but lately it has been shown to be highly vulnerable to cumulative lifetime stress [158] and therefore its acceleration can be a predictor of late life mortality risk [159]. Examination of *post mortem* brain tissue gives rather inconsistent results, mainly due to the different methodologies used in each study. A common point among the majority of these studies though is that methylation is not as enriched in promoter and active transcription sites as it is in intronic regions and long intergenic non-coding RNAs [154, 156]. Additionally, in one study, a high correlation between the transcriptome and the methylome was demonstrated [157], overall hinting at a more subtle regulatory function of methylation potentially linked with other epigenetic mechanisms such as microRNAs. Such theories can perspectivevely be tested and deeper understanding of the role of methylation changes in BP pathology can be provided with more extensive research on the methylation patterns across brain areas as well as within cell types. This thesis highly contributes to this cause by providing data from the first ever EWAS utilizing the EPIC array on *post mortem* cerebellum tissue.

The role of epigenetics in neurodegenerative and neurodevelopmental disorders

This empirical narrative vividly illustrates that the epigenetic machinery, and more specifically DNA modification processes, are involved in the pathophysiology of the aforementioned representative trio of disorders that are all characterized by cognitive, emotional, social and executive dysfunction. The exact role of DNA modifications however has yet to be elucidated. Nonetheless, more extensive research into the epigenetic mechanisms has paved the way to the formulation of theories that could finally close the “heritability gap”. Epigenetic mechanisms have three strong properties: (i) they can modulate gene transcription, (ii) they are an intercellular regulatory hub interconnecting genomics, epigenomics and transcriptomics, (iii) they can be transgenerationally inherited. Thus, with respect to the aetiopathogenesis of AD, SZ and BP, epigenetic mechanisms could convey the effect of environmental exposures into modified gene expression thus inducing a catastrophic cascade of intracellular events leading to pathology, or a dysregulated epigenetic machinery could either directly prompt aberrant transcription or modify the resilience to environmental stressors, making the individual more susceptible to developing the disease.

Whether epigenetic mechanisms are makers or markers of disease pathogenesis, is a critical answer that should soon be responded, if we wish to offer effective therapeutic and diagnostic alternatives and thus alleviate the socioeconomic burden that these diseases impose. The dawn of the affordable Illumina array technology era in combination with advanced bioinformatics analysis pipelines provide a unique opportunity for scientist worldwide to profile the peripheral and central epigenome. Subsequently, state-of-the-art methodologies such as epigenetic editing by means of CRISPR/Cas9 could provide an optimal platform in order to successfully define the role of DNA modification in the aetiopathogenesis of such disorders.

Overview of thesis

This thesis is a collection of research lines mainly merging towards profiling epigenetic modifications in three mental and neurodegenerative disorders, namely AD, SZ and BP. The first three chapters focus on the involvement of diverse epigenetic mechanisms in AD, while the fourth presents an EWAS in relation to SZ and BP. Finally the last chapter deals with a slightly different topic, still within the realms of AD pathology.

After a brief yet concise introduction on DNA modifications and the current *status quo* of their involvement on the pathology of specific neurological disorders, **CHAPTER 2** reviews the advancements in epigenomic technology and their application in AD-centered research. On the same chapter, questions of temporal and spatial causality of AD pathogenesis are raised and the brainstem is set in the spotlight as a structure of particular interest in the incipient stages of AD.

Following on that notion, **CHAPTER 3** sets the dorsal raphe nucleus (DRN) as the cornerstone for interrogating early-stage AD epigenetic signatures. State-of-the-art methodology is used and the methylome as well as the hydroxymethylome profile of the DRN is presented. Differentially methylated, hydroxymethylated, and unmodified positions as well as altered genomic regions are detected in relation to the progression of the disease as portrayed by the Braak stage escalation. Moreover, biological networks associated with aggravation of AD pathology were identified based on modules of co-methylated genomic positions.

CHAPTER 4 reflects a different approach on epigenetics in AD as it focuses on the interplay between methylation and expression of microRNAs (miRs). To this end, the central and peripheral methylome of *MIR137* is profiled and associated with AD diagnosis, as well as levels of $A\beta$ and tau pathology. Additionally, the expression levels of miR-137 were examined within a specific brain region of AD patients and age-matched controls.

In search of DNA methylation changes associated with psychotic-related phenotypes, **CHAPTER 5** presents an EWAS performed in cerebellar tissue of patients diagnosed with SZ and BP. The two disorders were examined jointly under the umbrella “major psychosis” but in isolation too. Common and distinct signatures were therefore identified and replicated by a second independent EWAS performed again in cerebellar tissue from relevant subjects from a different cohort.

The final study presented in **CHAPTER 6** takes a step away from epigenetic research while still focusing on AD. This research line represents an attempt to provide a therapeutic alternative to the main symptom of AD, memory impairment. The memory restoring properties of a synthetic agonist targeting the neurotrophin signaling system were therefore challenged in a mouse model of AD.

The key outcomes and findings of this thesis are summarized in **CHAPTER 7**. Overall strengths and limitations of this thesis are then presented along with critical points concerning mainly the research field of epigenetics. Finally, future perspectives are provided.

CYTOSINE STATES

DETECTION STRATEGIES

Figure 1: (A) Cycle of cytosine modifications. Unmethylated cytosine (UC) is converted into 5-methylcytosine (5mC) via DNA methyltransferases (DNMT). Consecutively, ten-eleven translocation enzymes (TET) catalyse the active demethylation of 5mC to 5-hydroxymethylation (5hmC), 5-fomylcytosine (5fC), and 5-carboxylcytosine (5caC). Finally, 5fC and 5caC are decarboxylised into UC by thymine–DNA–glycosylase (TDG)-catalysed base excision and DNA base excision repair (BER). (B) Overview of the procedure to detect unmodified cytosines (C), 5mC, and 5hmC. The DNA samples under interrogation are divided in two; the first half is treated with bisulfite (BS), and the second first gets oxidized (ox) and then undergoes the BS conversion. BS treatment of the first half converts the C into thymines (T) while all other modified C are protected against this conversion and thus are read as a C on the array. The extracted BS signal from this step is the combined signal from 5mC and 5hmC. Oxidation preceding the BS treatment converts the 5hmC to 5fC, retracting the protection of 5hmC to the action of BS conversion, and therefore turning it into T as well. To this end, oxBS signal represents only the fraction of 5mC. Therefore, the 5hmC fraction can be captured by subtracting the oxBS signal from the BS signal, and further the unmodified C fraction can be determined by subtracting the BS signal from 1.

References

1. Qiu, C., M. Kivipelto, and E. von Strauss, Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. *Dialogues in Clinical Neuroscience*, 2009. 11(2): p. 111-128.
2. Burmeister, M., M.G. McInnis, and S. Zöllner, Psychiatric genetics: progress amid controversy. *Nature Reviews Genetics*, 2008. 9(7): p. 527-540.
3. Pidsley, R., et al., A data-driven approach to preprocessing Illumina 450K methylation array data. *BMC Genomics*, 2013. 14(1): p. 293.
4. Aminoff, S.R., et al., Polygenic risk scores in bipolar disorder subgroups. *Journal of Affective Disorders*, 2015. 183: p. 310-314.
5. Waddington, C., Canalization of development and their inheritance of acquired characters. *Nature*, 1942b. 150: p. 563-565.
6. Russo, V.E.A.R., Arthur D & Martienssen, Robert A Introduction. In *Epigenetic mechanisms of gene regulation 1996*, Cold Spring Harbor Laboratory Press, Plainview, N.Y. p. 1-4.
7. Henikoff, S. and M.A. Matzke, Exploring and explaining epigenetic effects. *Trends Genet*, 1997. 13(8): p. 293-5.
8. Berger, S.L., et al., An operational definition of epigenetics. *Genes Dev*, 2009. 23(7): p. 781-3.
9. Felsenfeld, G., A Brief History of Epigenetics. *Cold Spring Harbor Perspectives in Biology*, 2014. 6(1): p. a018200.
10. Guo, J.U., et al., Distribution, recognition and regulation of non-CpG methylation in the adult mammalian brain. *Nat Neurosci*, 2014. 17(2): p. 215-22.
11. Portela, A. and M. Esteller, Epigenetic modifications and human disease. *Nat Biotechnol*, 2010. 28(10): p. 1057-68.
12. Ziller, M.J., et al., Charting a dynamic DNA methylation landscape of the human genome. *Nature*, 2013. 500(7463): p. 477-81.
13. Klose, R.J. and A.P. Bird, Genomic DNA methylation: the mark and its mediators. *Trends in Biochemical Sciences*, 2006. 31(2): p. 89-97.
14. Aran, D., et al., Replication timing-related and gene body-specific methylation of active human genes. *Hum Mol Genet*, 2011. 20(4): p. 670-80.
15. Ball, M.P., et al., Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol*, 2009. 27(4): p. 361-8.

16. Hellman, A. and A. Chess, Gene body-specific methylation on the active X chromosome. *Science*, 2007. 315(5815): p. 1141-3.
17. Lister, R., et al., Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, 2009. 462(7271): p. 315-22.
18. Rauch, T.A., et al., A human B cell methylome at 100-base pair resolution. *Proc Natl Acad Sci U S A*, 2009. 106(3): p. 671-8.
19. Flores, K., et al., Genome-wide association between DNA methylation and alternative splicing in an invertebrate. *BMC Genomics*, 2012. 13: p. 480.
20. Lyko, F., et al., The honey bee epigenomes: differential methylation of brain DNA in queens and workers. *PLoS Biol*, 2010. 8(11): p. e1000506.
21. Lev Maor, G., A. Yearim, and G. Ast, The alternative role of DNA methylation in splicing regulation. *Trends Genet*, 2015. 31(5): p. 274-80.
22. Marina, R.J., et al., TET-catalyzed oxidation of intragenic 5-methylcytosine regulates CTCF-dependent alternative splicing. *EMBO J*, 2015.
23. Roubroeks, J.A., et al., Epigenetics and DNA methylomic profiling in Alzheimer's disease and other neurodegenerative diseases. *Journal of neurochemistry*, 2017.
24. Guo, J.U., et al., Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell*, 2011. 145(3): p. 423-34.
25. Wu, H. and Y. Zhang, Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev*, 2011. 25(23): p. 2436-52.
26. Hashimoto, H., et al., Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. *Nucleic Acids Res*, 2012. 40(11): p. 4841-9.
27. Wen, L. and F. Tang, Genomic distribution and possible functions of DNA hydroxymethylation in the brain. *Genomics*, 2014. 104(5): p. 341-346.
28. Wen, L., et al., Whole-genome analysis of 5-hydroxymethylcytosine and 5-methylcytosine at base resolution in the human brain. *Genome Biol*, 2014. 15(3): p. R49.
29. Ito, S., et al., Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*, 2011. 333(6047): p. 1300-3.

30. Shin, J., G.-l. Ming, and H. Song, DNA modifications in the mammalian brain. *Phil. Trans. R. Soc. B*, 2014. 369(1652): p. 20130512.
31. Pfaffeneder, T., et al., The discovery of 5-formylcytosine in embryonic stem cell DNA. *Angewandte Chemie International Edition*, 2011. 50(31): p. 7008-7012.
32. Song, C.-X., et al., Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. *Cell*, 2013. 153(3): p. 678-691.
33. He, Y.-F., et al., Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science*, 2011. 333(6047): p. 1303-1307.
34. Ladd-Acosta, C., et al., DNA Methylation Signatures within the Human Brain. *American Journal of Human Genetics*, 2007. 81(6): p. 1304-1315.
35. Hsieh, J. and X. Zhao, *Genetics and Epigenetics in Adult Neurogenesis*. Cold Spring Harb Perspect Biol, 2016. 8(6).
36. Feng, J., et al., Dnmt1 and Dnmt3a are required for the maintenance of DNA methylation and synaptic function in adult forebrain neurons. *Nature neuroscience*, 2010. 13(4): p. 423-430.
37. Kaas, G.A., et al., TET1 Controls CNS 5-methylcytosine Hydroxylation, Active DNA Demethylation, Gene Transcription and Memory Formation. *Neuron*, 2013. 79(6): p. 10.1016/j.neuron.2013.08.032.
38. Zhang, R.-R., et al., Tet1 regulates adult hippocampal neurogenesis and cognition. *Cell stem cell*, 2013. 13(2): p. 237-245.
39. Szulwach, K.E., et al., 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nature neuroscience*, 2011. 14(12): p. 1607-1616.
40. Arnold, S.E., et al., Olfactory epithelium amyloid-beta and paired helical filament-tau pathology in Alzheimer disease. *Ann Neurol*, 2010. 67(4): p. 462-9.
41. Dugger, B.N., et al., The distribution of phosphorylated tau in spinal cords of Alzheimer's disease and non-demented individuals. *J Alzheimers Dis*, 2013. 34(2): p. 529-36.
42. Braak, H. and K. Del Tredici, The preclinical phase of the pathological process underlying sporadic Alzheimer's disease. *Brain*, 2015.
43. Braak, H. and K. Del Tredici, Amyloid-beta may be released from non-junctional varicosities of axons generated from abnormal tau-containing brainstem

nuclei in sporadic Alzheimer's disease: a hypothesis. *Acta Neuropathologica*, 2013. 126(2): p. 303-306.

44. Curcio, C.A. and T. Kemper, Nucleus raphe dorsalis in dementia of the Alzheimer type: neurofibrillary changes and neuronal packing density. *J Neuropathol Exp Neurol*, 1984. 43(4): p. 359-68.

45. German, D.C., C.L. White, 3rd, and D.R. Sparkman, Alzheimer's disease: neurofibrillary tangles in nuclei that project to the cerebral cortex. *Neuroscience*, 1987. 21(2): p. 305-12.

46. Aston-Jones, G. and J.D. Cohen, An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annu Rev Neurosci*, 2005. 28: p. 403-50.

47. Dringenberg, H.C., Alzheimer's disease: more than a 'cholinergic disorder' - evidence that cholinergic-monoaminergic interactions contribute to EEG slowing and dementia. *Behav Brain Res*, 2000. 115(2): p. 235-49.

48. Grinberg, L.T., et al., The dorsal raphe nucleus shows phospho-tau cytoskeletal changes before the transentorhinal region in AD. A new (or earlier) beginning? *Acta Neuropathologica*, 2008. 116(3): p. 343-343.

49. O'Donnell, J., et al., Norepinephrine: a neuromodulator that boosts the function of multiple cell types to optimize CNS performance. *Neurochem Res*, 2012. 37(11): p. 2496-512.

50. Braak, H. and K. Del Tredici, Where, when, and in what form does sporadic Alzheimer's disease begin? *Current Opinion in Neurology*, 2012. 25(6): p. 708-714.

51. Braak, H., et al., Stages of the Pathologic Process in Alzheimer Disease: Age Categories From 1 to 100 Years. *Journal of Neuropathology and Experimental Neurology*, 2011. 70(11): p. 960-969.

52. Simic, G., et al., Annotation - Does Alzheimer's disease begin in the brainstem? *Neuropathology and applied neurobiology*, 2009. 35(6): p. 532-554.

53. Arendt, T., J. Stieler, and M. Holzer, Brain hypometabolism triggers PHF-like phosphorylation of tau, a major hallmark of Alzheimer's disease pathology. *J Neural Transm (Vienna)*, 2015. 122(4): p. 531-9.

54. Chalermpananupap, T., et al., Targeting norepinephrine in mild cognitive impairment and Alzheimer's disease. *Alzheimers Res Ther*, 2013. 5(2): p. 21.

55. Grudzien, A., et al., Locus coeruleus neurofibrillary degeneration in aging, mild cognitive impairment and early Alzheimer's disease. *Neurobiol Aging*, 2007. 28(3): p. 327-35.
56. Haglund, M., M. Sjoberck, and E. Englund, Locus ceruleus degeneration is ubiquitous in Alzheimer's disease: possible implications for diagnosis and treatment. *Neuropathology*, 2006. 26(6): p. 528-32.
57. Lyness, S.A., C. Zarow, and H.C. Chui, Neuron loss in key cholinergic and aminergic nuclei in Alzheimer disease: a meta-analysis. *Neurobiol Aging*, 2003. 24(1): p. 1-23.
58. Marcyniuk, B., D.M. Mann, and P.O. Yates, The topography of cell loss from locus caeruleus in Alzheimer's disease. *J Neurol Sci*, 1986. 76(2-3): p. 335-45.
59. Marien, M.R., F.C. Colpaert, and A.C. Rosenquist, Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev*, 2004. 45(1): p. 38-78.
60. Mesulam, M., et al., Cholinergic nucleus basalis tauopathy emerges early in the aging-MCI-AD continuum. *Ann Neurol*, 2004. 55(6): p. 815-28.
61. Trillo, L., et al., Ascending monoaminergic systems alterations in Alzheimer's disease. translating basic science into clinical care. *Neurosci Biobehav Rev*, 2013. 37(8): p. 1363-79.
62. Weinshenker, D., Functional consequences of locus coeruleus degeneration in Alzheimer's disease. *Curr Alzheimer Res*, 2008. 5(3): p. 342-5.
63. Zweig, R.M., et al., Neuropathology of aminergic nuclei in Alzheimer's disease. *Prog Clin Biol Res*, 1989. 317: p. 353-65.
64. Busch, C., J. Bohl, and T.G. Ohm, Spatial, temporal and numeric analysis of Alzheimer changes in the nucleus coeruleus. *Neurobiol Aging*, 1997. 18(4): p. 401-6.
65. Parvizi, J., G.W. Van Hoesen, and A. Damasio, The selective vulnerability of brainstem nuclei to Alzheimer's disease. *Ann Neurol*, 2001. 49(1): p. 53-66.
66. Sara, S.J., The locus coeruleus and noradrenergic modulation of cognition. *Nat Rev Neurosci*, 2009. 10(3): p. 211-23.
67. Schliebs, R. and T. Arendt, The cholinergic system in aging and neuronal degeneration. *Behav Brain Res*, 2011. 221(2): p. 555-63.

68. Theofilas, P., et al., Turning on the Light Within: Subcortical Nuclei of the Isodentritic Core and their Role in Alzheimer's Disease Pathogenesis. *Journal of Alzheimers Disease*, 2015. 46(1): p. 17-34.
69. Elobeid, A., H. Soininen, and I. Alafuzoff, Hyperphosphorylated tau in young and middle-aged subjects. *Acta Neuropathologica*, 2012. 123(1): p. 97-104.
70. Arendt, T., et al., Early neurone loss in Alzheimer's disease: cortical or subcortical? *Acta Neuropathol Commun*, 2015. 3: p. 10.
71. Bhatia, N. and G. Hall, Untangling the role of tau in Alzheimer's disease: A unifying hypothesis. *Translational Neuroscience*, 2013. 4(2): p. 115-133.
72. Czech, C., G. Tremp, and L. Pradier, Presenilins and Alzheimer's disease: biological functions and pathogenic mechanisms. *Prog Neurobiol*, 2000. 60(4): p. 363-84.
73. De Strooper, B., et al., Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, 1998. 391(6665): p. 387-90.
74. Goate, A., Segregation of a missense mutation in the amyloid beta-protein precursor gene with familial Alzheimer's disease. *J Alzheimers Dis*, 2006. 9(3 Suppl): p. 341-7.
75. Hardy, J.A. and G.A. Higgins, Alzheimer's disease: the amyloid cascade hypothesis. *Science*, 1992. 256(5054): p. 184-5.
76. Lardenoije, R., et al., The epigenetics of aging and neurodegeneration. *Prog Neurobiol*, 2015. 131: p. 21-64.
77. Migliore, L. and F. Coppede, Genetics, environmental factors and the emerging role of epigenetics in neurodegenerative diseases. *Mutat Res*, 2009. 667(1-2): p. 82-97.
78. Sherrington, R., et al., Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, 1995. 375(6534): p. 754-60.
79. Tanzi, R.E., et al., The gene defects responsible for familial Alzheimer's disease. *Neurobiol Dis*, 1996. 3(3): p. 159-68.
80. Lemere, C.A., et al., The E280A presenilin 1 Alzheimer mutation produces increased A beta 42 deposition and severe cerebellar pathology. *Nat Med*, 1996. 2(10): p. 1146-50.

81. Gandhi, S. and N.W. Wood, Genome-wide association studies: the key to unlocking neurodegeneration? *Nat Neurosci*, 2010. 13(7): p. 789-94.
82. Harold, D., et al., Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet*, 2009. 41(10): p. 1088-93.
83. Hollingworth, P., et al., Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nat Genet*, 2011. 43(5): p. 429-35.
84. Naj, A.C., et al., Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat Genet*, 2011. 43(5): p. 436-41.
85. Lambert, J.C., et al., The CALHM1 P86L polymorphism is a genetic modifier of age at onset in Alzheimer's disease: a meta-analysis study. *J Alzheimers Dis*, 2010. 22(1): p. 247-55.
86. Slioter, A.J., et al., Risk estimates of dementia by apolipoprotein E genotypes from a population-based incidence study: the Rotterdam Study. *Arch Neurol*, 1998. 55(7): p. 964-8.
87. Kim, J., et al., Apolipoprotein E in Synaptic Plasticity and Alzheimer's Disease: Potential Cellular and Molecular Mechanisms. *Molecules and Cells*, 2014. 37(11): p. 767-776.
88. Suri, S., et al., The forgotten APOE allele: A review of the evidence and suggested mechanisms for the protective effect of APOE ε2. *Neuroscience & Biobehavioral Reviews*, 2013. 37(10, Part 2): p. 2878-2886.
89. Weingarten, M.D., et al., A protein factor essential for microtubule assembly. *Proc Natl Acad Sci U S A*, 1975. 72(5): p. 1858-62.
90. Ballatore, C., V.M. Lee, and J.Q. Trojanowski, Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci*, 2007. 8(9): p. 663-72.
91. Ksiazek-Reding, H. and S.-H. Yen, Structural stability of paired helical filaments requires microtubule-binding domains of Tau: A model for self-association. *Neuron*, 1991. 6(5): p. 717-728.
92. Novak, M., et al., Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51. *Proceedings of the National Academy of Sciences of the United States of America*, 1991. 88(13): p. 5837-5841.

93. Crowther, R.A., et al., The microtubule binding repeats of tau protein assemble into filaments like those found in Alzheimer's disease. *FEBS Lett*, 1992. 309(2): p. 199-202.
94. Crary, J.F., et al., Primary age-related tauopathy (PART): a common pathology associated with human aging. *Acta Neuropathol*, 2014. 128(6): p. 755-66.
95. Sanders, D.W., et al., Distinct tau prion strains propagate in cells and mice and define different tauopathies. *Neuron*, 2014. 82(6): p. 1271-88.
96. Klafki, H.W., et al., Therapeutic approaches to Alzheimer's disease. *Brain*, 2006. 129(Pt 11): p. 2840-55.
97. Lee, V.M., M. Goedert, and J.Q. Trojanowski, Neurodegenerative tauopathies. *Annu Rev Neurosci*, 2001. 24: p. 1121-59.
98. Mastroeni, D., et al., Epigenetic mechanisms in Alzheimer's disease. *Neurobiol Aging*, 2011. 32(7): p. 1161-80.
99. Mastroeni, D., et al., Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. *PLoS One*, 2009. 4(8): p. e6617.
100. Mufson, E.J., et al., Braak staging, plaque pathology, and APOE status in elderly persons without cognitive impairment. *Neurobiology of Aging*, 2016. 37: p. 147-153.
101. Chouliaras, L., et al., Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. *Neurobiology of aging*, 2013. 34(9): p. 2091-2099.
102. Condliffe, D., et al., Cross-region reduction in 5-hydroxymethylcytosine in Alzheimer's disease brain. *Neurobiology of aging*, 2014. 35(8): p. 1850-1854.
103. Mastroeni, D., et al., Epigenetic changes in Alzheimer's disease: decrements in DNA methylation. *Neurobiology of aging*, 2010. 31(12): p. 2025-2037.
104. Bradley-Whitman, M. and M. Lovell, Epigenetic changes in the progression of Alzheimer's disease. *Mechanisms of ageing and development*, 2013. 134(10): p. 486-495.
105. Ellison, E.M., E.L. Abner, and M.A. Lovell, Multiregional analysis of global 5-methylcytosine and 5-hydroxymethylcytosine throughout the progression of Alzheimer's disease. *Journal of neurochemistry*, 2017. 140(3): p. 383-394.
106. West, R.L., J.M. Lee, and L.E. Maroun, Hypomethylation of the amyloid precursor protein gene in the brain of an Alzheimer's disease patient. *Journal of Molecular Neuroscience*, 1995. 6(2): p. 141-146.

107. Iwata, A., et al., Altered CpG methylation in sporadic Alzheimer's disease is associated with APP and MAPT dysregulation. *Human molecular genetics*, 2013. 23(3): p. 648-656.
108. Barrachina, M. and I. Ferrer, DNA methylation of Alzheimer disease and tauopathy-related genes in postmortem brain. *Journal of Neuropathology & Experimental Neurology*, 2009. 68(8): p. 880-891.
109. Brohede, J., et al., A DNA methylation study of the amyloid precursor protein gene in several brain regions from patients with familial Alzheimer disease. *Journal of neurogenetics*, 2010. 24(4): p. 179-181.
110. Bakulski, K.M., et al., Genome-wide DNA methylation differences between late-onset Alzheimer's disease and cognitively normal controls in human frontal cortex. *Journal of Alzheimer's Disease*, 2012. 29(3): p. 571-588.
111. Sanchez-Mut, J.V., et al., Promoter hypermethylation of the phosphatase DUSP22 mediates PKA-dependent TAU phosphorylation and CREB activation in Alzheimer's disease. *Hippocampus*, 2014. 24(4): p. 363-368.
112. De Jager, P.L., et al., Alzheimer's disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDL2 and other loci. *Nature neuroscience*, 2014. 17(9): p. 1156.
113. Lunnon, K., et al., Methylomic profiling implicates cortical deregulation of ANK1 in Alzheimer's disease. *Nature neuroscience*, 2014. 17(9): p. 1164.
114. Watson, C.T., et al., Genome-wide DNA methylation profiling in the superior temporal gyrus reveals epigenetic signatures associated with Alzheimer's disease. *Genome medicine*, 2016. 8(1): p. 5.
115. Patel, K.R., et al., Schizophrenia: Overview and Treatment Options. *Pharmacy and Therapeutics*, 2014. 39(9): p. 638-645.
116. LAVRETSKY, H., History of schizophrenia as a psychiatric disorder. *Clinical handbook of schizophrenia*, 2008: p. 1.
117. Os, J.v., "Schizophrenia" does not exist. *BMJ*, 2016. 352.
118. Gejman, P.V., A.R. Sanders, and J. Duan, The Role of Genetics in the Etiology of Schizophrenia. *The Psychiatric clinics of North America*, 2010. 33(1): p. 35-66.
119. Maric, N. and D. Svrakic, Why schizophrenia genetics needs epigenetics: a review. *Psychiatria Danubina*, 2012. 24(1.): p. 2-18.

120. Abdolmaleky, H.M., et al., Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *Human molecular genetics*, 2006. 15(21): p. 3132-3145.

121. Abdolmaleky, H.M., et al., Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2005. 134(1): p. 60-66.

122. Iwamoto, K., et al., DNA methylation status of SOX10 correlates with its downregulation and oligodendrocyte dysfunction in schizophrenia. *Journal of Neuroscience*, 2005. 25(22): p. 5376-5381.

123. Zhang, A.P., et al., The DNA methylation profile within the 5'-regulatory region of DRD2 in discordant sib pairs with schizophrenia. *Schizophr Res*, 2007. 90(1-3): p. 97-103.

124. Mill, J., et al., Epigenomic Profiling Reveals DNA-Methylation Changes Associated with Major Psychosis. *American Journal of Human Genetics*, 2008. 82(3): p. 696-711.

125. Pidsley, R., et al., Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology*, 2014. 15(10): p. 483.

126. Viana, J., et al., Schizophrenia-associated methylomic variation: molecular signatures of disease and polygenic risk burden across multiple brain regions. *Human Molecular Genetics*, 2017. 26(1): p. 210-225.

127. Jiang, T., et al., Variation in global DNA hydroxymethylation with age associated with schizophrenia. *Psychiatry Res*, 2017. 257: p. 497-500.

128. Health, N.C.C.f.M. Bipolar disorder: the management of bipolar disorder in adults, children and adolescents, in primary and secondary care. 2006. British Psychological Society.

129. Manji, H.K., et al., The underlying neurobiology of bipolar disorder. *World Psychiatry*, 2003. 2(3): p. 136-146.

130. Konradi, C., S.E. Sullivan, and H.B. Clay, Mitochondria, oligodendrocytes and inflammation in bipolar disorder: evidence from transcriptome studies points to intriguing parallels with multiple sclerosis. *Neurobiology of disease*, 2012. 45(1): p. 37-47.

131. Cardno, A.G. and M.J. Owen, Genetic Relationships Between Schizophrenia, Bipolar Disorder, and Schizoaffective Disorder. *Schizophrenia Bulletin*, 2014. 40(3): p. 504-515.

132. Aminoff, S.R., et al., Polygenic risk scores in bipolar disorder subgroups. *Journal of Affective Disorders*. 183: p. 310-314.
133. Kerner, B., Toward a Deeper Understanding of the Genetics of Bipolar Disorder. *Frontiers in Psychiatry*, 2015. 6: p. 105.
134. Oliveira, J., et al., *Toxoplasma gondii* exposure may modulate the influence of TLR2 genetic variation on bipolar disorder: a gene-environment interaction study. *International Journal of Bipolar Disorders*, 2016. 4: p. 11.
135. Aldinger, F. and T.G. Schulze, Environmental factors, life events, and trauma in the course of bipolar disorder. *Psychiatry and clinical neurosciences*, 2017. 71(1): p. 6-17.
136. D'Addario, C., et al., Selective DNA methylation of BDNF promoter in bipolar disorder: differences among patients with BDI and BDII. *Neuropsychopharmacology*, 2012. 37(7): p. 1647-55.
137. Rao, J.S., et al., Epigenetic modifications in frontal cortex from Alzheimer's disease and bipolar disorder patients. *Transl Psychiatry*, 2012. 2: p. e132.
138. Dell'Osso, B., et al., Epigenetic modulation of BDNF gene: differences in DNA methylation between unipolar and bipolar patients. *J Affect Disord*, 2014. 166: p. 330-3.
139. Strauss, J.S., et al., Quantitative leukocyte BDNF promoter methylation analysis in bipolar disorder. *Int J Bipolar Disord*, 2013. 1: p. 28.
140. Stenz, L., et al., BDNF promoter I methylation correlates between post-mortem human peripheral and brain tissues. *Neurosci Res*, 2015. 91: p. 1-7.
141. Carrard, A., et al., Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. *J Affect Disord*, 2011. 132(3): p. 450-3.
142. Ghadirivasfi, M., et al., Hypomethylation of the serotonin receptor type-2A Gene (HTR2A) at T102C polymorphic site in DNA derived from the saliva of patients with schizophrenia and bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet*, 2011. 156B(5): p. 536-45.
143. Perroud, N., et al., Methylation of Serotonin Receptor 3a in Adhd, Borderline Personality, and Bipolar Disorders: Link with Severity of the Disorders and Childhood Maltreatment. *Depress Anxiety*, 2016. 33(1): p. 45-55.
144. Tamura, Y., et al., Epigenetic aberration of the human REELIN gene in psychiatric disorders. *Mol Psychiatry*, 2007. 12(6): p. 519, 593-600.

145. Fatemi, S.H., Reelin, a marker of stress resilience in depression and psychosis. *Neuropsychopharmacology*, 2011. 36(12): p. 2371-2.
146. Abdolmaleky, H.M., et al., Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *Hum Mol Genet*, 2006. 15(21): p. 3132-45.
147. Burghardt, K.J., et al., DNA methylation, insulin resistance and second-generation antipsychotics in bipolar disorder. *Epigenomics*, 2015. 7(3): p. 343-52.
148. Dempster, E.L., et al., Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder. *Hum Mol Genet*, 2011. 20(24): p. 4786-96.
149. Houtepen, L.C., et al., DNA methylation signatures of mood stabilizers and antipsychotics in bipolar disorder. *Epigenomics*, 2016. 8(2): p. 197-208.
150. Li, Y., et al., Genome-wide methylome analyses reveal novel epigenetic regulation patterns in schizophrenia and bipolar disorder. *Biomed Res Int*, 2015. 2015: p. 201587.
151. Sabunciyen, S., et al., Association of DNA Methylation with Acute Mania and Inflammatory Markers. *PLoS One*, 2015. 10(7): p. e0132001.
152. Walker, R.M., et al., DNA methylation in a Scottish family multiply affected by bipolar disorder and major depressive disorder. *Clin Epigenetics*, 2016. 8: p. 5.
153. Fries, G., et al., Integrated transcriptome and methylome analysis in youth at high risk for bipolar disorder: a preliminary analysis. *Translational psychiatry*, 2017. 7(3): p. e1059.
154. Zhao, H., et al., Genome-wide DNA methylome reveals the dysfunction of intronic microRNAs in major psychosis. *BMC Med Genomics*, 2015. 8: p. 62.
155. Mill, J., et al., Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. *Am J Hum Genet*, 2008. 82(3): p. 696-711.
156. Xiao, Y., et al., The DNA methylome and transcriptome of different brain regions in schizophrenia and bipolar disorder. *PLoS One*, 2014. 9(4): p. e95875.
157. Chen, C., et al., Correlation between DNA methylation and gene expression in the brains of patients with bipolar disorder and schizophrenia. *Bipolar Disord*, 2014. 16(8): p. 790-9.

158. Zannas, A.S., et al., Lifetime stress accelerates epigenetic aging in an urban, African American cohort: relevance of glucocorticoid signaling. *Genome biology*, 2015. 16(1): p. 266.
159. Marioni, R.E., et al., DNA methylation age of blood predicts all-cause mortality in later life. *Genome biology*, 2015. 16(1): p. 25.

2

Epigenetic dysregulation of brainstem nuclei in the pathogenesis of Alzheimer's disease: looking in the correct place at the right time?

Abstract	p.38
Keywords	p.38
Background	p.39
Epigenomic technology advancements in AD	p.40
Towards genome-wide sequencing of the AD brain	p.41
Beyond DNA and CpG specific methylation	p.42
Cross regional and blood differences in epigenetic modifications	p.43
Cell-specific epigenetic changes	p.43
Sample size caveat; loophole through validation cohorts?	p.44
Integrative genetic and epigenetic analyses	p.45
Brainstem: where it all starts?	p.46
The raphe nuclei and AD	p.48
The locus coeruleus and AD	p.50
Looking to the future	p.51
Conclusion	p.53
Acknowledgements	p.53
References	p.54

Iatrou A¹, Kenis G¹, Rutten BPF¹, Lunnon K^{2*}, van den Hove DLA^{1,3*†}

1 School for Mental Health and Neuroscience (MHeNS), Department of Psychiatry and Neuropsychology, Maastricht University, Universiteitssingel 50, 6200 MD Maastricht, The Netherlands.

2 University of Exeter Medical School, RILD, University of Exeter, Barrack Road, Devon, UK.

3 Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Fuechleinstrasse 15, 97080 Wuerzburg, Germany

*Authors contributed equally to this work

† Corresponding author: e-mail: d.vandenhove@maastrichtuniversity.nl; Tel: +31-43-3882203; Fax: +31-43-3884086

Cellular and Molecular Life Sciences 74.3, 509-523 (2017)

Abstract

Even though the aetiology of Alzheimer's disease (AD) remains unknown, it is suggested that interplay among genetic, epigenetic and environmental factors is involved. An increasing body of evidence pinpoints that dysregulation in the epigenetic machinery plays a role in AD. Recent developments in genomic technologies have allowed for high throughput interrogation of the epigenome, and epigenome-wide association studies (EWAS) have already identified unique epigenetic signatures for AD in the cortex. Considerable evidence suggests that early dysregulation in the brainstem, more specifically in the raphe nuclei and the locus coeruleus, accounts for the most incipient, non-cognitive symptomatology, indicating a potential causal relationship with the pathogenesis of AD. Here we review the advancements in epigenomic technologies and their application to the AD research field, particularly with relevance to the brainstem. In this respect, we propose the assessment of epigenetic signatures in the brainstem as the cornerstone of interrogating causality in AD. Understanding how epigenetic dysregulation in the brainstem contributes to AD susceptibility could be of pivotal importance for understanding the aetiology of the disease and for the development of novel diagnostic and therapeutic strategies.

Keywords: Alzheimer's disease; Locus Coeruleus; Raphe Nuclei; Epigenetics; DNA methylation; DNA hydroxymethylation

Background

Alzheimer's disease (AD) is a chronic, neurodegenerative disorder that currently accounts for 60-80% of dementia cases [1,2]. The prevalence of AD is expected to increase dramatically with the exponential increase in the aging population and a lack of effective therapeutic options. Recent evidence suggests that the incipient stages of the disease may start in young adulthood where they remain asymptomatic until advanced age [3,4]. Throughout its progression, AD deprives patients of their quality of life, by negatively impacting upon emotional control, cognition, memory, and language skills, converting them to highly dependent reflections of their past selves, and substantially decreasing their life expectancy. The pathogenesis of AD is associated with amyloid beta ($A\beta$) plaques, which form degradation-resistant aggregates, and hyperphosphorylated tau protein that leads to the formation of intraneuronal neurofibrillary tangles (NFTs) [2,5]. These two characteristic hallmarks are believed to lead to synaptic dysfunction and eventually neuronal cell loss, causing dramatic cortical and subcortical atrophy [6-9]. While the hypotheses about the preliminary appearance of one of the two hallmarks are raging, a definite mechanism has yet to be provided (i.e. [10,8]). To date, the $A\beta$ burden has mainly been associated with the neurobiological underpinning of AD whereas tau pathology is positively correlated with the progression of cognitive deterioration in the patients [10,11,3].

In AD, individual disease risk is determined by genetic and environmental factors, as well as complex interactions between them. From a genetic perspective AD can be classified into two subtypes, familial or sporadic, and while the symptomatology and the progression of both forms are comparable, the etiology is fundamentally different [12]. Familial AD accounts for only 5-10% of the disease cases and is related to the existence of genetic mutations in specific genes, such as those encoding amyloid precursor protein (APP) and presenilin (PSEN) 1 (PSEN1) and PSEN2 [13-20], which are all involved in the production of $A\beta$. Sporadic AD is the most prevalent form of AD, usually occurs later in life (> 65 years) and bares non-Mendelian traits. In recent years, common genetic variants have been robustly associated with sporadic AD via genome-wide association studies (GWAS) and subsequent meta-analyses ([21]; for specific GWAS results see [22-26]), although these only account for a third of disease susceptibility risk [21]. Therefore, more recent research efforts have focused on a potential role for epigenetic mechanisms in disease etiology [27].

To date, even though there is a strong association between hallmark appearance and the incidence of AD, the pathogenesis of the disease remains uncertain. Moreover, evidence has shown that some individuals may carry the most salient genetic risk factors for AD and also express profuse $A\beta$ and tau pathology, but yet never develop the disorder [17,28-30]. Strikingly, even monozygotic twins can

have discordant AD outcomes [29], and as such it has been suggested that these phenomena could be explained by epigenetic mechanisms [27]. The epigenetic machinery induces reversible changes in gene expression via covalent interactions with mainly the chromatin components. These modifications in gene activity, while ever-changing, are more pronounced during development and remain more stable in differentiated cell types. Hence, normal dynamic changes in the epigenetic machinery are responsible for cellular development and differentiation, but also for transiently imprinting environmental, behavioral as well as social effects on gene expression, maintaining genomic homeostasis throughout the lifespan. The umbrella term, epigenetic modifications, covers a gamut of mechanisms, namely DNA modifications (5-methylcytosine [5-mC], 5-hydroxymethylcytosine [5-hmC], 5-formylcytosine [5-fC], and 5-carboxylcytosine [5-caC]), chromatin remodeling by means of remodeling complexes and post-translational histone modifications, and non-coding RNA (ncRNAs; long ncRNA [lncRNA], short ncRNA [sncRNA]). Currently, the best-characterized epigenetic modifications are DNA modifications, with DNA methylation within CpG islands being the most extensively studied. Contrary to popular belief, DNA methylation is not solely associated with gene repression, but the differential effect on gene activity depends on the location of the epigenetic modification on the gene or its proximity [31]. Additionally, the newly characterized DNA modifications, 5-hmC, 5-fC, 5-caC, were originally thought to be transient marks in the demethylation pathway; however, recent evidence suggests that 5-hmC may represent an independent epigenetic mark and has been associated with active gene transcription [32]. In AD, recent epigenome-wide association studies (EWAS) have identified robust changes in DNA methylation patterns in specific genes; yet whether this remains a cause or a consequence of the disease is not currently known.

This review provides a thorough update on the fast-pacing advancements in (epi) genomic technology with a main focus on its application to AD-related research. Moreover, by reviewing recent evidence on the early involvement of the brainstem in the non-cognitive early symptomatology of the disease, it discusses the need to systematically assess epigenetic dysregulation in this brain region to identify novel dysfunctional pathways. Ultimately, this review aims to raise critical questions of temporal and spatial causality of AD pathogenesis and how the answer may be found in innovative brain structure targets with the assistance of state-of-the-art genomic technology.

Epigenomic technology advancements in AD

Over the past decade, the number of publications investigating the role of epigenetic mechanisms in AD has dramatically increased, which have substantially contributed to our understanding of the disease (reviewed by Lardenoije et al. [17]). Major advances in genomic technology have helped overcome numerous

hurdles that were faced in the early years of neuroepigenetic studies [27]. Such caveats involved the limited available techniques, the specificity and reliability of the epigenetic methodology used, as well as issues concerning genomic coverage, tissue cell-type composition, and sample sizes.

Towards genome-wide sequencing of the AD brain

It is evident that genomic studies in AD have now progressed from restricted, targeted antibody-based techniques to genome-wide arrays and sequencing technology with single CpG site resolution. In 1995, the first empirical studies in AD used methylation-specific restriction enzymes and Southern Blot technique demonstrated hypomethylation in the APP gene promoter region [33]. Since then, several approaches have emerged, involving immunohistochemistry, methylation-specific polymerase chain reaction (PCR), bisulfite (BS) conversion, high performance liquid chromatography (HPLC), pyrosequencing and various methylation assays [34]. The imperative need for more concise and collective results shifted epigenetic research in AD to more systematic genome-wide approaches. In 2012, Baluski and colleagues were the first to utilize Illumina microarray technology, Infinium HumanMethylation27 Beadchip assay, enabling quantification of DNA methylation at > 27,000 CpG sites, and detected AD-associated DNA methylation differences in the prefrontal cortex of late-onset sporadic AD patients in comparison to cognitively normal controls [35]. More recently, studies have employed the more extensive, current workhorse for epigenetic studies, the Infinium HumanMethylation450 BeadChip assay (450K), detecting > 485,000 methylation sites at a single-nucleotide resolution, covering approximately 1.5% of total genomic CpG sites, mainly amidst promoter regions [36,37,26,38,39]. The first-two large-scale EWAS in AD identified overlapping differentially methylated CpG loci, namely ANK1, RPL13, CDH23, and RHBDF2 [40]. This year, a further Illumina Infinium microarray (Illumina MethylationEPIC Beadarray) was launched, covering > 850,000 CpG sites [41], although it has yet to be utilized in AD. The continuous advancements in microarray technology, combined with their cost effectiveness have made this approach the most widely utilized EWAS method in large sample cohort studies. However, such methodologies only cover a small percentage of CpG sites and thus whole-genome sequencing techniques remain the best option for in-depth genome-wide examination. Only recently, the first whole-genome bisulfite sequencing (WGBS) was conducted with much wider coverage than just the promoter proximal CpGs (20% of total genomic CpG sites) [42]. In addition, the first low(er)-cost deep sequencing reduced representation bisulfite sequencing (RRBS) kit recently became available with a high coverage of up to 4,000,000 CpGs in human samples [43], and as such the use if these technologies in AD tissue is anticipated.

Beyond DNA and CpG specific methylation

Epigenomic studies have been largely focused on DNA (cytosine) methylation, overlooking additional epigenetic signatures. To date, further methodological improvements have allowed the detection of demethylation marks (5-hmC, 5-fC, 5-caC), post-translational histone modifications as well as deregulated ncRNAs. New advancements have allowed the discrimination of 5-hmC and 5-fC by employing oxidative bisulfite sequencing (oxBS-Seq) and reduced bisulfite sequencing (redBS-Seq), respectively [44-47]. More recently, researchers have made use of this chemistry and coupled it with the 450K array, presenting the oxBS-450K method [48]. This method was used successfully to identify differential DNA hydroxymethylation patterns across different anatomical regions of the human brain but also and most importantly to accurately quantify “true” methylation levels that up until now were confounded by hydroxymethylation levels [49]. Application of oxBS-arrays or oxBS sequencing on human AD samples will hopefully not only highlight the importance of DNA demethylation in cognitive processes but also confirm the hypothesized crucial role of 5-hmC in AD as hinted by immunohistochemical studies [50-53] (reviewed in [54]). Whilst for the DNA modifications 5-fC and 5-caC, there are currently only two studies examining their levels in AD with disparate results; Condliffe et al. [51] did not detect AD-associated differences whereas Bradley-Whitman et al. reported a decrease in the hippocampal area in preclinical AD samples [55]. Therefore, studies using redBS-Seq or comparable techniques will elucidate the levels of these modifications in AD at single nucleotide resolution.

While traditional epigenetic research has focused on methylation of a cytosine within a CpG dinucleotide, largely within CpG islands, more recent studies have begun examining intermediate/low CG-content regions as well as non-CpG DNA methylation. An increasing number of targeted AD studies nowadays examine the methylation status of more than just CpG-rich gene promoter areas [38]. The newly developed WGBS method provides adequate information about intergenic CpGs distal to gene promoters as well as non-CpG methylation [42]. Thus, it is expected that implementation of this technique in AD studies will contribute to a deeper understanding of DNA methylation to the pathophysiology and will highlight further regions on the genome that display differential DNA methylation in disease.

Although genome-wide histone modification analysis using chromatin immunoprecipitation (ChIP) techniques are available, to date no studies have used this approach in AD. In fact, only three published studies have directly connected histone modifications to AD. Zhang and colleagues found downregulated H3K18 and H3K23 acetylation when comparing temporal lobe samples from AD patients to those of controls by using monitoring liquid chromatography-mass

spectrometry [56]. Mastroeni and colleagues immunohistochemically detected aberrant extra-nuclear localization of H3K4 tri-methylation at the most incipient stages of the disease [57]. Whilst, Graff and colleagues detected increase in the protein levels of histone deacetylase 2 (HDAC2) in AD brains [58]. Finally, while micro-RNAs (miRNAs) are very well studied with targeted and genome-wide array-based methods, other ncRNAs have been generally understudied. Recently though, AD-associated lncRNAs have been identified for the first time by re-annotating previously probed uniquely mapped lncRNAs [59]. Among the most significantly dysregulated lncRNAs were n341006 and n336934, lncRNAs involved in protein ubiquitination and cholesterol homeostasis respectively [59].

Cross regional and blood differences in epigenetic modifications

One caveat when examining epigenetic as opposed to genetic variation is the need to investigate changes in a tissue-specific manner. To date, studies have largely utilized tissue from various cortical regions given that these are the site of neurodegeneration and dysfunction observed with advanced progression of the disease (hippocampus, frontal cortex dorsolateral prefrontal cortex, the entorhinal cortex, the superior temporal cortex, the medio-temporal gyrus, the superior temporal gyrus), although a handful have also included the cerebellum [38,51,36,37]. The use of cerebellum is rather interesting in such studies as it is relatively spared from AD pathology, even at the late stages of the disease, and thus serves as an internal control tissue.

The investigation of tissue-specific epigenetic signatures in the brain allows the elucidation of the underlying mechanisms in the pathophysiology of AD, whilst interrogation of epigenetic variation in the blood is of pivotal importance in order to develop novel molecular biomarkers for the early diagnosis of AD. To date only a handful of studies have investigated DNA methylation changes in blood from AD patients; D'Addario's team showed global DNA hypermethylation in blood samples of sporadic AD patients, while Lunnon et al. detected DNA methylation differences at specific loci in ante-mortem blood samples from sporadic AD patients. Altogether, these results encourage further research to identify AD-related epigenetic signatures as biomarkers in larger sample cohorts.

Cell-specific epigenetic changes

While the identification of AD-related epigenetic changes in post-mortem brain tissue is highly important for a better understanding of the pathophysiology, the cellular heterogeneity constitutes a major caveat in interpreting the results. It is well described in the literature that AD-related neurodegeneration is highly specific towards selected neuronal cell types and is also accompanied by glial activation,

which could confound the interpretation of epigenetic studies on brain tissue in AD. Steps to specify cell type composition can be taken in early experimental stages with a range of methods available to isolate specific cell types. Such methods involve density gradients, laser capture microdissection (LCM), fluorescent-activated cell sorting (FACS), magnetic affinity cell sorting (MACS) and, more recently, isolation of nuclei tagged in specific cell types (INTACT) [60,61]. The INTACT method is specifically adapted for interrogating epigenetic marks ranging from DNA methylation to histone modifications by means of selectively capturing nuclei that express an antibody-tagged protein [60]. Hence, its application will be very fruitful for unravelling neuron or glial specific AD-related epigenetic signatures. LCM has recently been employed for the characterization of amyloid plaques [62] as well as gene expression via RNA sequencing comparing AD and control brain tissue [63]. Encouraging data from the latter study imply that LCM could be used for specific cell type isolation in epigenetic studies, since it does not appear to induce disease-unrelated transcriptional changes. Interesting applications of this methodology would be not only targeting the epigenetic profiling of neurons either in the vicinity of AD hallmarks (namely gliosis and amyloid plaques), or severely affected by tau pathology, but also assessing the differential epigenetic signatures of AD-pathology spared neurons. Finally, in already collected datasets on unsorted tissue, bioinformatic analyses can also correct for neuronal/glia composition utilizing published algorithms [64]. This approach has already been used in the analysis of 450K array data generated in AD tissue enabling the researchers to control for cellular heterogeneity bias [37,38].

Sample size caveat; loophole through validation cohorts?

Regardless of the technological improvements that have assisted a deeper investigation of the epigenetic machinery in AD, there is still one caveat that persists; the sample size of the cohorts used. To date, there is a circumscribed amount of EWAS studies on AD [36-38,65,66,35], and only two of them have a sample size exceeding 100 [36,37]. A fortunate phenomenon in the limited number of EWAS studies in AD, performed to date, is that a considerable number of epigenetic alterations have a replicable effect in independent cohorts from other studies (i.e. finding from [37] have been replicated by [36,38]). The falling cost of whole-genome studies in combination with the exponential increase in high quality brain tissue available from brain banks worldwide will probably reinforce studies with larger sample size. Nevertheless, one should be cautious with the predilection of tissue from AD patients. It was recently suggested that DNA methylation profiles of various neurodegenerative disorders, including AD, involve similar early epigenetic-associated pathogenic mechanisms, which, over time evolve into divergent clinical cases with distinct molecular and cellular underpinnings [65]. This concept was also supported by the latent early-life

associated regulation (LEARn) model of Lahiri and colleagues which proposes that neurobiological disorders share a similar mechanistic etiology [67]. More specifically, according to this hypothesis, early life stressors modify the expression levels of disorder-associated genes, a change that is transiently maintained by epigenetic mechanisms and is shared among a wider spectrum of neurobiological disorders. The differential expression of these genes remains within physiological range, until, later in life, multiple “hits”, i.e. environmental agents, dietary factors, and lifestyle habits, accumulate, leading to aberrant-pathological changes in expression [67,68]. Evidently, these notions should be taken into consideration for longitudinal studies in populations at high risk of developing AD [69]. Further, research on incipient stages of AD might not be in conflict with other confounding factors, but as the pathology worsens, the epigenome seems to change dramatically. Moreover, recent evidence showed that once proper bioinformatic analysis is employed - i.e. correcting not only for technical issues and sex, but also for common neuropathologies seen in the elderly population - the total number of age-dependent CpG methylation profiles is reduced by approximately 40% [70]. Therefore, large sample sizes with a thorough medical history of medication, information on concurrent neuropathologies as well as epigenetic-modifying environmental exposures, together with the reciprocal advances in bioinformatic analysis tools, would hone current EWAS studies in AD.

Integrative genetic and epigenetic analyses

Even from the restricted number of EWAS studies thus far published, a common locus, BIN1 [36,26], is found to overlap with GWAS results, leading the way for integrated analysis of genomic and epigenomic data that could essentially address causality in AD (for a thorough review see [71]). It has already been shown that genetic variants can influence DNA methylation [72]. In this respect, application of the Mendelian randomization (MR) method could strengthen the causal assumption, and help in elucidating the interplay among genetic variation, epigenetic modifications and environmental factors. For example, with the recently described two-step epigenetic MR method, firstly the causal impact of a risk factor in an epigenetic modification is interrogated using a genetic variant as intermediate for the risk factor, and then the causal effect of the investigated epigenetic change, is examined on the desired outcome (i.e. AD) [73]. To date, EWAS data can assist identifying the risk factor-epigenetic modification association at the first step and GWAS data can provide the genetic variant proxy. Interestingly, with the identification and study of methylation quantitative trait loci (mQTL) in the human brain [74], it will be possible to trace SNPs associated with methylation at specific genomic regions and use them as proxy [73]. An additional integrative analysis of genomic, epigenomic and enviromic data called longitudinal epigenome/envirome/exposome-wide association study (LEWAS) was suggested by Lahiri and Maloney [75]. The rationale of this approach is the

combination of genomic information with repeatedly-collected information of the patient's envirome and the epigenome [76,75]. Therefore, changes in epigenetic markers could be linked to the transient changes measured prior to the clinical manifestation of a disease [75]. All these approaches would allow the exploration of new disease mechanisms in order to ultimately start to answer the question: "Is epigenetic dysregulation a cause or consequence of AD?" Nevertheless, at this point, it is important to note that while the MR approach is feasible practically, LEWAS remains a rather theoretical method due to the in-depth interrogation of the patient's environmental exposures as well as the high costs it would require to conduct such a study [reviewed by Maloney and Lahiri [76]].

One key issue that is yet to be addressed is the temporal and spatial causality of AD pathogenesis, for example whether the "state-of-the-art" technology that is being applied in the field is being done so in relevant brain regions at appropriate time points. Nowadays, ante-mortem AD diagnosis is mainly based on cognitive deficits associated with hippocampal and cortical dysfunction, as well as with imaging studies, primarily focused on the size of the hippocampus, which also makes these brain regions primary targets for GWAS and EWAS studies. Despite the catalytic involvement of hippocampal neurodegeneration and dysfunction in the progression of the disease, it is speculated that once the pathology has reached these structures, the deleterious effects on brain integrity are already irreversible [77]. Therefore, the interrogation of (epi)genetic modifications at that stage mainly contributes to a mechanistic understanding of the progression rather than the cause of the disease. A full mapping of epigenetic changes in a range of different brain structures at the appropriate stage(s) of disease is more likely to offer insight into the disease course, from the initial stages to the extensive neurodegeneration of cortical and subcortical areas. Such an approach could prompt early-stage biomarkers and novel therapeutic targets for the most incipient stages of the disease, in addition to providing predictive models for the expansion of the disease.

Brainstem: where it all starts?

Human AD pathology is primarily confined to the central nervous system (CNS) [78,79]. There, the pathology propagates in a rather predictable, selective spatial and temporal manner with some regions being highly vulnerable to the aforementioned hallmarks at specific stages and others relatively resistant [7]. It is remarkable that the pathology vastly targets very specific neuronal types, which share long, late-myelinating and weakly myelinating axons [7,8]. Thus, the earliest detection of abnormal hyperphosphorylated tau protein has been observed in the brainstem, and more specifically in the magnocellular nuclei of the basal forebrain, the raphe nuclei and the locus coeruleus (LC) [7,8,80-86,3,87-103,4,104]. From there, pathology propagates to highly vulnerable subcortical

areas, i.e. the entorhinal cortex and hippocampus, and, subsequently, to high-order association areas of the neocortex [85,7,105]. Once cortical areas are affected, the curtailment of intellectual functions begins, gradually leading to deterioration or even loss of executive functions, annotating the clinical phase of AD.

The well-orchestrated propagation of hallmarks from subcortical to cortical regions has allowed staging of the various preclinical and clinical phases of AD and has facilitated the definition of neuropathological diagnostic criteria. Among the most widely used are the modified criteria based on NFT propagation described by Braak and Braak [106]. The original staging scheme of 1997 included four stages: Braak stage 0 (no NFTs); Braak stages I/II, with NFTs amidst the (trans) entorhinal cortex area; Braak stages III/IV, with NFTs expanding over to the hippocampus and the amygdala as well as cortical areas; and Braak stages V/VI, with pronounced NFTs over the isocortex [107]. In 2011, the aforementioned scheme was updated with the addition of the preclinical stages a-c and 1a-1b. Indicative of stages a-c is non-fibrillar abnormal tau pathology in the brainstem, mainly becoming traceable during teenage years [3]. Stages 1a-1b concern cases with early abnormal tau pathology at pyramidal cells in the transentorhinal cortex. Finally, extensive research complements the Braak staging providing associations with $A\beta$ pathology measurements as well as AD clinical assessment tools, i.e. the mini-mental stage examination (MMSE) [7].

Increasing evidence that the brainstem may be the starting point of the propagation of AD pathology has triggered an ever-increasing scientific interest in the involvement of the brainstem in AD and numerous studies have investigated a central role of the brain serotonergic and noradrenergic systems in its pathophysiology (reviewed by [103,7,87]). The brainstem in AD patients was recently shown to be subjected not only to significant volume reductions, but also structural deformations in a magnetic resonance imaging (MRI) study [108]. Further, the early occurrence of various non-cognitive, behavioral and neuropsychological symptoms in AD, such as depression, general disturbances in mood, emotion, appetite, respiratory and circadian rhythm, suggests brainstem involvement, and more specifically that of the raphe nuclei and the LC [87,3,109]. Moreover, brainstem nuclei are affected by AD pathology, particularly tangles, in very early, presymptomatic stages [3,110,103,87,111,7]. Interestingly, despite their vulnerability to tau pathology, the death of NFT-bearing neurons is not imminent during the presymptomatic stage and even at the final stages these neurons seem to be more resilient to degeneration [112-114,99,115,92,103,7]. Nevertheless, at that time, their function is highly impaired, impacting on the brain's neurochemical balance [103].

The raphe nuclei and AD

The raphe nuclei, and in particular the dorsal raphe nucleus (DRN) contains long projecting neurons that are abundant in serotonin (5-hydroxytryptamine; 5-HT), a monoamine neurotransmitter synthesized out of tryptophan [116]. The serotonergic system has been implicated in almost every type of basic physiological behavior, including appetite, sleep, emotional, cognitive as well as motor and neuroendocrine functions [116]. This widely distributed network in the brain mainly innervates the prosencephalon, including key areas for cognitive function, such as the frontal cortex, hippocampus, striatum, hypothalamus and amygdala [117,118].

One study has found that more than 20% of Braak stage 0 individuals and 100% of Braak stage 1 individuals have detectable NFTs in the DRN, indicating that the DRN is affected by AD pathology even before the transentorhinal cortex [119]. Accordingly, it has been suggested that the development of pathology in the brainstem might trigger a transneuronal spread of NFTs changes to interconnected cortical brain areas affected at later Braak stages [110]. Even if tau “seeding” is still poorly understood, a suggested mechanism is that, once released, intracellularly formed tau aggregates extracellularly and is transferred to neighboring cells, thereby inducing the production of abnormal tau at those sites [120]. Several hypotheses on the formation and propagation of neurotoxic $A\beta$ species have ensued from this hypothesized “seeding” effect. In particular, Braak and Del Tredici suggested that $A\beta$ may originate from projection neurons with abnormal tau within the brainstem nuclei. Observations of accumulated toxic $A\beta$ species in the vicinity of somatodendritic compartments of neurons as well as in the terminals of their axons in brain structures well-innervated by NFT-bearing 5-HT/NA projections could justify the fine pattern of $A\beta$ propagation and suggests that toxic $A\beta$ species are produced and released from such projection neurons [8]. Nevertheless, this hypothesis remains to be tested.

The severity of AD pathology in the DRN has been correlated not only with serotonergic denervation but interestingly also with behavioral changes in AD patients [104,103,121]. For example, the NFT-associated lesions that are present in the DRN even in the early phase, are largely held responsible for explaining mood symptoms such as depression and aggression, in prodromal AD [104]. Plaque and NFT load in the DRN and the median raphe nuclei (MRN) of AD patients has been shown to correlate with the progression of clinical symptoms [121,122]. Additionally, a dysregulated serotonergic system has been linked not only to cognitive decline, but also to disturbances in the circadian rhythm seen in prodromal AD stages [123,124].

From an anatomical point of view, post-mortem immunohistochemically stained AD brain samples (Braak stage V and VI) have shown a decreased number of serotonergic neurons in the DRN and the MRN [103]. This observation was recently replicated and enhanced with correlation analysis that exhibited an age-dependent 5-HT cell loss in particular nuclei [103,7]. Interestingly, there seems to be a predilection for neurodegeneration in the caudal part of the DRN, which predominantly projects to the septum and the hippocampal area [98,121]. Supporting evidence for the involvement of the 5-HT system in AD has been provided by imaging studies. Positron emission tomography (PET) studies have found that 5-HT_{1A} receptors were reduced in the hippocampi and raphe nuclei and that the decrease was strongly correlated with deterioration in the MMSE scores [125]. Moreover, while in MCI patients a hippocampus-specific increase in 5-HT metabolism and receptors (5-HT_{1A}) has been observed, in advanced stages of AD, serotonergic receptors are dramatically downregulated in cortical areas [103]. Interestingly, functional genetic coding variants in the brain-specific tryptophan hydroxylase-2 (TPH2) and the 5-HT transporter (5-HTT) have been significantly associated with frontal lobe symptoms in AD [126].

Complementary research on the role of the serotonergic system in AD has yielded interesting results concerning 5-HT system function and AD pathology. Preclinical studies have demonstrated that an increase of 5-HT levels via e.g. pharmacological activation attenuates A β pathology. Both acute and chronic administration of selective serotonin reuptake inhibitors (SSRIs) induces reduction in the production of toxic A β species in brains of APP^{swe}/PS1^{dE9} mice, a widely used AD mouse model [127,128]. The acute administration of SSRIs is directly impacting on A β synthesis rather than the clearance rate of the plaques, the main mechanism of action of the ineffective drug bapineuzumab, a humanized monoclonal antibody targeting A β , developed for the treatment of AD [129]. In addition, intrahippocampal infusion of 5-HT as well as dietary enrichment of tryptophan in the same AD mouse model is associated with a reduction in the formation of A β plaques. Furthermore, treatment of non-cognitive impaired elderly participants with SSRIs for 5 consecutive years has been associated with less cortical amyloid deposition as revealed by a positron emission tomography (PET) study. Moreover, a reduction in the production as well as levels of A β was detected in the cerebrospinal fluid (CSF) of healthy volunteers treated with citalopram, a commonly used SSRI [128]. Finally, while 5-HT_{1A}, 5-HT₄ and 5-HT₆ receptor ligands are known to modify cognitive functions [130], they were also shown to favor the production of non-amyloidogenic A β precursors that do not aggregate, with administration of 5-HT₄ receptor agonists increasing the levels of soluble APP (sAPP-A) [131].

The locus coeruleus and AD

The LC is the principal site for brain synthesis of noradrenaline (NA). NA is a catecholamine synthesized from tyrosine by a series of enzymatic steps that lead to the formation of dopamine, which is finally converted to the final product by dopamine beta-hydroxylase (DBH) [103]. Similar to the 5-HT system, the NA system consists of long projection neurons that are widely distributed throughout the brain [8]. Nevertheless, patterns of regional specificity arise as the frontal and parietal cortices are richly innervated [132]. Functionally, NA has been implicated in wakefulness and attention as well as the endocrine response to stress, while more recent evidence has also linked it to cognition, pain, aggression as well as energy homeostasis and blood flow control [132].

With regard to the role of the LC in AD, Braak and colleagues revealed that tau pathology is present in the LC prior to any other structure and even before any clinical symptoms or amyloid pathology manifestation was evident [3]. Histopathological observations of the LC using post-mortem AD brains have identified signs of atrophy, including swollen cell bodies, contracted dendrites and significantly decreased detection of NAergic markers [133,112,90,134,135,103,91,105]. The deformation of the LC and the associated impairment in NA neurotransmission have been linked to the onset, severity, disease duration, speed of cognitive decline as well as with the appearance of AD pathology [103]. Concerning the latter, loss of NA neurons has been associated with increased A β deposition as well as an increased amount of cortical NFTs, strongly supporting the seeding-like propagation pattern previously suggested [8].

The noradrenergic system appears to be highly dysregulated in AD. Post-mortem studies have demonstrated reduced NA synthesis and availability in the frontal and temporal cortex as well as in the hippocampal area [136,137,90]. The rate of reduction in NA levels has been positively correlated with the severity of AD [133,136,138,139]. Additionally, Vermeiren and colleagues showed significantly decreased levels of 3-methoxy-4-hydroxyphenylglycol (MHPG), a metabolite of NA degradation, in the prefrontal cortex of AD patients suffering from depression [140]. Nevertheless, other studies have reported an increase in NA and MHPG plasma and CSF levels solely in advanced AD cases [141,142]. Another study did replicate the increased NA concentration in the CSF but failed to detect changes in the levels of MHPG [143]. Notably, one should be cautious interpreting results from CSF studies to brain function, as NA normally cannot cross the blood brain barrier (BBB), while its metabolites like MHPG, are able to do so [144]. Thus, CSF concentrations of MHPG reflect a sum of central and peripheral levels and are unlikely to reflect the most conclusive markers of the disease progression [145].

Studies have also indicated a decrease in DBH activity in the neocortex and the hippocampus of post-mortem tissue from patients at early AD stages [146]. This observation has been recently replicated by Mustapic and colleagues who additionally showed a gradual decrease in enzymatic activity with the progression of the disease and deterioration of cognitive functions [147]. Further, the reported decrease could explain the decreased NA levels and the loss of noradrenergic neurons [147].

Restoration of NA levels via e.g. exercise or pharmacological manipulation has shown beneficial effects on cognition in AD. Segal and colleagues demonstrated that exercise-mediated activation of the NAergic system can enhance memory consolidation in MCI patients and controls [148]. Moreover, administration of L-threo-dihydroxyphenylserine (L-DOPS), a prodrug for NA, enhances contextual and recognition memory in NA-deficient mouse models. Moreover, once administered to AD mouse models, restoration of spatial memory performance as well as a reduction in amyloid plaque number and size in the cortex and hippocampus were observed [149,150].

Looking to the future

Despite the increasing interest in brainstem dysfunction in AD, it still remains poorly understood whether the previously described structural, chemical, and functional alterations are causally involved in the pathogenesis of AD or whether they merely represent a consequence of the deleterious progression of the disease, or an epiphenomenon. The appearance of tau pathology with the early non-cognitive symptomatology suggests that the brainstem may reflect the initial structure affected by AD pathology in the brain. Bearing in mind the functional importance of the raphe nuclei and the LC as signaling hubs of top-down neuromodulatory input to high-order cortical areas as well as their vulnerability to AD neuropathology, it is tempting to speculate that they have a crucial role in the etiopathogenesis of AD. Furthermore, the lack of genetic attributes interlacing dysregulations in brainstem-specific neurotransmission with AD progression or pathology may furthermore hint at an environmental and/or epigenetic involvement. Indeed, both the nuclei have been repeatedly investigated as targets of epigenetic control in various developmental stages as well as in neurological disorders. The noradrenergic developmental genes of the LC for instance have been reported to be under epigenetic control suggesting the vulnerability of the nucleus to environmental input [151,152]. Moreover, it has been previously shown that functioning of the i.e. 5-HT system is sensitive to gene-environment interactions (e.g. stress)[153-156]. Thus the sensitivity of both the nuclei to environmental stimuli and epigenetic

regulation in combination with the evidence that the brainstem is one of the first structures to present AD pathology and that robust epigenetic changes are seen in the latter affected cortical regions in AD offer fertile ground for further research in studying epigenetic dysfunction in the brainstem in the most incipient stages of AD.

The complex and yet elusive nature of sporadic AD allows for various hypotheses to explain the pathogenesis of the disease. To date, none of these hypotheses have been confirmed despite the advancements in genomic technology that provide deeper insight in to the molecular underpinnings of AD. A possible reason is the temporal discrepancies between the biological and the clinical onset of the disease. Furthermore, the majority of research studies focus on brain structures vastly associated with the clinical phase of the disease thereby overlooking the preclinical manifestations of pathology. Such studies are invaluable in enriching the fundamental knowledge about the pathophysiology during the progression of AD, but it is unlikely that they will result in any of the two imperative societal needs: early, reliable, non-invasive and inexpensive biomarkers and effective treatment options that target the disease in its most incipient stages, much earlier than the first manifestations of cognitive curtailment.

Peripheral disease-associated epigenetic signatures have already been successfully employed as diagnostic tools for different cancers, and are currently being studied in neurological/psychiatric disorders [157-160]. As mentioned above, the advancements in genomic technology allows for high throughput interrogation of the epigenome and the extensive study of the correlation between brain and blood epigenetic signatures will contribute to the emergence of non-invasive and inexpensive biomarkers. Thus, epigenetic profiling of the brainstem of (sporadic) AD patients and its pairing with blood epigenetic signatures in the same individuals could potentially lead to the discovery of novel biomarkers that are able to detect either subtle changes at the very early stages of the disease, when the pathology is believed to be still reversible, or even an early peripheral response to AD pathology. Yet another exceedingly valuable asset of the study of epigenetic dysregulation in the brainstem is the fact that various pharmacological interventions impacting on either the epigenetic machinery ([161]; reviewed by Maloney and Lahiri [76]) or the 5-HT/NA system have already been developed and clinically approved; hence they could be implemented rapidly as a novel intervention for AD. Collectively, scrutinizing the interactions between the early AD-affected brainstem and the local epigenetic modifications will be of pivotal importance not only for understanding the pathogenesis of AD and the causal or consequential relationship of epigenetic alterations with AD, but also for the development of highly-demanded early, reliable biomarkers and novel therapeutic strategies.

Conclusion

The preclinical manifestations of AD, governed by non-clinical symptoms, suggest a crucial involvement of brainstem nuclei in the pathophysiology and most importantly in the pathogenesis of AD. Meanwhile, distinct, yet consistent, epigenetic signatures emerging from EWAS studies indicate a central role for the epigenetic machinery in the progression of AD. To date, while the exponential increase of AD-related research lines offers disparate interpretations in the cause of AD, the lack of effective diagnostic and/or therapeutic tools suggest that the etiology of the disease remains shrouded. With this review we wish to perturb the status quo of AD, that is the genomic and epigenomic interrogation of brain regions like the hippocampus and cortex. We suggest that the temporal and spatial manifestations of the disease should be aligned and thus advocate that the two key nodes of the early stages of AD should be scrutinized. The pairing of brainstem pathology with deviant epigenetic regulation, indicative of the incipient stages of AD pathology, could serve as excellent candidate targets for further research that could lead to the development on early biomarkers as well as early treatment alternatives that could halt or even reverse the deleterious progression of AD.

Acknowledgements

Funds have been provided by the Joint Programme - Neurodegenerative Disease Research (JPND) for the EPI-AD consortium focusing on epigenetic dysregulation in the brainstem in Alzheimer's Disease (http://www.neurodegenerationresearch.eu/wp-content/uploads/2015/10/Factsheet_EPI-AD.pdf). The project is supported through the following funding organizations under the aegis of JPND - www.jpnd.eu, The Netherlands, The Netherlands Organisation for Health Research and Development (ZonMw); United Kingdom, Medical Research Council; Germany, German Federal ministry of Education and Research (BMBF); Luxembourg, National Research Fund (FNR). This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 643417. Additional support has been provided by the UK Medical Research Council (MRC) grant MR/N027973/1 (KL), Alzheimer's Association (US) New Investigator Research Grant NIRG-14-320878 (KL), Alzheimer's Society (UK) grant AS-PG-14-038 (KL), the Internationale Stichting Alzheimer Onderzoek (ISAO) grants 07551 and 11532 (D.LvdH.), the ISAO grant 12530 (G.K), the ISAO grant 13515 (B.P.F. R), and the Netherlands Organization for Scientific Research (NWO) grant 916.11.086 (Veni Award) (B.P.F. R). The authors declare that they have no conflict of interest.

References

1. Guo JU, Su Y, Shin JH, Shin J, Li H, Xie B, Zhong C, Hu S, Le T, Fan G, Zhu H, Chang Q, Gao Y, Ming GL, Song H (2014) Distribution, recognition and regulation of non-CpG methylation in the adult mammalian brain. *Nature neuroscience* 17 (2):215-222. doi:10.1038/nn.3607
2. Selkoe DJ (2012) Preventing Alzheimer's Disease. *Science* 337 (6101):1488-1492
3. Braak H, Thal DR, Ghebremedhin E, Del Tredici K (2011) Stages of the Pathologic Process in Alzheimer Disease: Age Categories From 1 to 100 Years. *Journal of Neuropathology and Experimental Neurology* 70 (11):960-969. doi:10.1097/NEN.0b013e318232a379
4. Elobeid A, Soininen H, Alafuzoff I (2012) Hyperphosphorylated tau in young and middle-aged subjects. *Acta Neuropathol* 123 (1):97-104. doi:10.1007/s00401-011-0906-z
5. Mann DA, Hardy J (2013) Amyloid or tau: the chicken or the egg? *Acta Neuropathol* 126 (4):609-613. doi:10.1007/s00401-013-1162-1
6. Hardy J, Selkoe DJ (2002) The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science* 297 (5580):353-356. doi:10.1126/science.1072994
7. Braak H, Del Tredici K (2015) The preclinical phase of the pathological process underlying sporadic Alzheimer's disease. *Brain*. doi:10.1093/brain/awv236
8. Braak H, Del Tredici K (2013) Amyloid-beta may be released from non-junctional varicosities of axons generated from abnormal tau-containing brainstem nuclei in sporadic Alzheimer's disease: a hypothesis. *Acta Neuropathol* 126 (2):303-306. doi:10.1007/s00401-013-1153-2
9. Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. *Science* 298 (5594):789-791. doi:10.1126/science.1074069
10. Stancu IC, Ris L, Vasconcelos B, Marinangeli C, Goeminne L, Laporte V, Haylani LE, Couturier J, Schakman O, Gailly P, Pierrot N, Kienlen-Campard P, Octave JN, Dewachter I (2014) Tauopathy contributes to synaptic and cognitive deficits in a murine model for Alzheimer's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 28 (6):2620-2631. doi:10.1096/fj.13-246702

11. Attems J, Jellinger KA (2013) Amyloid and tau: neither chicken nor egg but two partners in crime! *Acta Neuropathol* 126 (4):619-621. doi:10.1007/s00401-013-1167-9
12. Bhatia N, Hall G (2013) Untangling the role of tau in Alzheimer's disease: A unifying hypothesis. *TranslatNeurosci* 4 (2):115-133. doi:10.2478/s13380-013-0114-5
13. Czech C, Tremp G, Pradier L (2000) Presenilins and Alzheimer's disease: biological functions and pathogenic mechanisms. *Prog Neurobiol* 60 (4):363-384
14. De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391 (6665):387-390. doi:10.1038/34910
15. Goate A (2006) Segregation of a missense mutation in the amyloid beta-protein precursor gene with familial Alzheimer's disease. *Journal of Alzheimer's disease : JAD* 9 (3 Suppl):341-347
16. Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256 (5054):184-185
17. Lardenoije R, Iatrou A, Kenis G, Kompotis K, Steinbusch HW, Mastroeni D, Coleman P, Lemere CA, Hof PR, van den Hove DL, Rutten BP (2015) The epigenetics of aging and neurodegeneration. *Prog Neurobiol* 131:21-64. doi:10.1016/j.pneurobio.2015.05.002
18. Migliore L, Coppede F (2009) Genetics, environmental factors and the emerging role of epigenetics in neurodegenerative diseases. *Mutation research* 667 (1-2):82-97. doi:10.1016/j.mrfmmm.2008.10.011
19. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Pericak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St George-Hyslop PH (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375 (6534):754-760. doi:10.1038/375754a0
20. Tanzi RE, Kovacs DM, Kim TW, Moir RD, Guenette SY, Wasco W (1996) The gene defects responsible for familial Alzheimer's disease. *Neurobiology of disease* 3 (3):159-168

21. Gandhi S, Wood NW (2010) Genome-wide association studies: the key to unlocking neurodegeneration? *Nature neuroscience* 13 (7):789-794. doi:10.1038/nn.2584
22. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Morgan K, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Love S, Kehoe PG, Hardy J, Mead S, Fox N, Rossor M, Collinge J, Maier W, Jessen F, Schurmann B, Heun R, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frolich L, Hampel H, Hull M, Rujescu D, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Muhleisen TW, Nothen MM, Moebus S, Jockel KH, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nature genetics* 41 (10):1088-1093. doi:10.1038/ng.440
23. Hollingworth P, Harold D, Sims R, Gerrish A, Lambert JC, Carrasquillo MM, Abraham R, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Jones N, Stretton A, Thomas C, Richards A, Ivanov D, Widdowson C, Chapman J, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Beaumont H, Warden D, Wilcock G, Love S, Kehoe PG, Hooper NM, Vardy ER, Hardy J, Mead S, Fox NC, Rossor M, Collinge J, Maier W, Jessen F, Ruther E, Schurmann B, Heun R, Kolsch H, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frolich L, Hampel H, Gallacher J, Hull M, Rujescu D, Giegling I, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Muhleisen TW, Nothen MM, Moebus S, Jockel KH, Klopp N, Wichmann HE, Pankratz VS, Sando SB, Aasly JO, Barcikowska M, Wszolek ZK, Dickson DW, Graff-Radford NR, Petersen RC, Alzheimer's Disease Neuroimaging I, van Duijn CM, Breteler MM, Ikram MA, DeStefano AL, Fitzpatrick AL, Lopez O, Launer LJ, Seshadri S, consortium C, Berr C, Campion D, Epelbaum J, Dartigues JF, Tzourio C, Alperovitch A, Lathrop M, consortium E, Feulner TM, Friedrich P, Riehle C, Krawczak M, Schreiber S, Mayhaus M, Nicolhaus S, Wagenpfeil S, Steinberg S, Stefansson H, Stefansson K, Snaedal J, Bjornsson S, Jonsson PV, Chouraki V,

Genier-Boley B, Hiltunen M, Soininen H, Combarros O, Zelenika D, Delepine M, Bullido MJ, Pasquier F, Mateo I, Frank-Garcia A, Porcellini E, Hanon O, Coto E, Alvarez V, Bosco P, Siciliano G, Mancuso M, Panza F, Solfrizzi V, Nacmias B, Sorbi S, Bossu P, Piccardi P, Arosio B, Annoni G, Seripa D, Pilotto A, Scarpini E, Galimberti D, Brice A, Hannequin D, Licastro F, Jones L, Holmans PA, Jonsson T, Riemenschneider M, Morgan K, Younkin SG, Owen MJ, O'Donovan M, Amouyel P, Williams J (2011) Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nature genetics* 43 (5):429-435. doi:10.1038/ng.803

24. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buross J, Gallins PJ, Buxbaum JD, Jarvik GP, Crane PK, Larson EB, Bird TD, Boeve BF, Graff-Radford NR, De Jager PL, Evans D, Schneider JA, Carrasquillo MM, Ertekin-Taner N, Younkin SG, Cruchaga C, Kauwe JS, Nowotny P, Kramer P, Hardy J, Huentelman MJ, Myers AJ, Barmada MM, Demirci FY, Baldwin CT, Green RC, Rogaeve E, St George-Hyslop P, Arnold SE, Barber R, Beach T, Bigio EH, Bowen JD, Boxer A, Burke JR, Cairns NJ, Carlson CS, Carney RM, Carroll SL, Chui HC, Clark DG, Corneveaux J, Cotman CW, Cummings JL, DeCarli C, DeKosky ST, Diaz-Arrastia R, Dick M, Dickson DW, Ellis WG, Faber KM, Fallon KB, Farlow MR, Ferris S, Frosch MP, Galasko DR, Ganguli M, Gearing M, Geschwind DH, Ghetti B, Gilbert JR, Gilman S, Giordani B, Glass JD, Growdon JH, Hamilton RL, Harrell LE, Head E, Honig LS, Hulette CM, Hyman BT, Jicha GA, Jin LW, Johnson N, Karlawish J, Karydas A, Kaye JA, Kim R, Koo EH, Kowall NW, Lah JJ, Levey AI, Lieberman AP, Lopez OL, Mack WJ, Marson DC, Martiniuk F, Mash DC, Masliah E, McCormick WC, McCurry SM, McDavid AN, McKee AC, Mesulam M, Miller BL, Miller CA, Miller JW, Parisi JE, Perl DP, Peskind E, Petersen RC, Poon WW, Quinn JF, Rajbhandary RA, Raskind M, Reisberg B, Ringman JM, Roberson ED, Rosenberg RN, Sano M, Schneider LS, Seeley W, Shelanski ML, Slifer MA, Smith CD, Sonnen JA, Spina S, Stern RA, Tanzi RE, Trojanowski JQ, Troncoso JC, Van Deerlin VM, Vinters HV, Vonsattel JP, Weintraub S, Welsh-Bohmer KA, Williamson J, Woltjer RL, Cantwell LB, Dombroski BA, Beekly D, Lunetta KL, Martin ER, Kamboh MI, Saykin AJ, Reiman EM, Bennett DA, Morris JC, Montine TJ, Goate AM, Blacker D, Tsuang DW, Hakonarson H, Kukull WA, Foroud TM, Haines JL, Mayeux R, Pericak-Vance MA, Farrer LA, Schellenberg GD (2011) Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nature genetics* 43 (5):436-441. doi:10.1038/ng.801

25. Lambert JC, Sleegers K, Gonzalez-Perez A, Ingelsson M, Beecham GW, Hiltunen M, Combarros O, Bullido MJ, Brouwers N, Bettens K, Berr C, Pasquier F, Richard F, Dekosky ST, Hannequin D, Haines JL, Tognoni G, Fievet N, Dartigues JF, Tzourio C, Engelborghs S, Arosio B, Coto E, De Deyn P, Del Zompo M, Mateo I, Boada M, Antunez C, Lopez-Arrieta J, Epelbaum J, Schjeide BM, Frank-Garcia A, Giedraitis V, Helisalmi S, Porcellini E, Pilotto A, Forti P, Ferri R, Delepine M, Zelenika D, Lathrop M, Scarpini E, Siciliano G, Solfrizzi V, Sorbi S, Spalletta G, Ravaglia G, Valdivieso F, Vepsalainen S, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossu P, Hanon O, Piccardi P, Annoni G, Mann D, Marambaud P, Seripa D, Galimberti D, Tanzi RE, Bertram L, Lendon C, Lannfelt L, Licastro F, Campion D, Pericak-Vance MA, Soininen H, Van Broeckhoven C, Alperovitch A, Ruiz A, Kamboh MI, Amouyel P (2010) The CALHM1 P86L polymorphism is a genetic modifier of age at onset in Alzheimer's disease: a meta-analysis study. *Journal of Alzheimer's disease* : JAD 22 (1):247-255. doi:10.3233/JAD-2010-100933
26. Yu L, Chibnik LB, Srivastava GP, Pochet N, Yang J, Xu J, Kozubek J, Obholzer N, Leurgans SE, Schneider JA, Meissner A, De Jager PL, Bennett DA (2015) Association of Brain DNA methylation in SORL1, ABCA7, HLA-DRB5, SLC24A4, and BIN1 with pathological diagnosis of Alzheimer disease. *JAMA neurology* 72 (1):15-24. doi:10.1001/jamaneurol.2014.3049
27. Lunnon K, Mill J (2013) Epigenetic studies in Alzheimer's disease: current findings, caveats, and considerations for future studies. *Am J Med Genet B Neuropsychiatr Genet* 162B (8):789-799. doi:10.1002/qjmg.b.32201
28. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, Rogers J (2011) Epigenetic mechanisms in Alzheimer's disease. *Neurobiology of aging* 32 (7):1161-1180. doi:10.1016/j.neurobiolaging.2010.08.017
29. Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD (2009) Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. *PLoS One* 4 (8):e6617. doi:10.1371/journal.pone.0006617
30. Mufson EJ, Malek-Ahmadi M, Perez SE, Chen K (2016) Braak staging, plaque pathology, and APOE status in elderly persons without cognitive impairment. *Neurobiology of aging* 37:147-153. doi:http://dx.doi.org/10.1016/j.neurobiolaging.2015.10.012
31. Ziller MJ, Gu H, Muller F, Donaghey J, Tsai LT, Kohlbacher O, De Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, Meissner A (2013) Charting a dynamic DNA methylation landscape of the human genome. *Nature* 500 (7463):477-481. doi:10.1038/nature12433

32. Wen L, Tang F (2014) Genomic distribution and possible functions of DNA hydroxymethylation in the brain. *Genomics* 104 (5):341-346. doi:10.1016/j.ygeno.2014.08.020

33. West RL, Lee JM, Maroun LE (1995) Hypomethylation of the amyloid precursor protein gene in the brain of an Alzheimer's disease patient. *Journal of Molecular Neuroscience* 6 (2):141-146

34. Kurdyukov S, Bullock M (2016) DNA Methylation Analysis: Choosing the Right Method. *Biology* 5 (1):3

35. Bakulski KM, Dolinoy DC, Sartor MA, Paulson HL, Konen JR, Lieberman AP, Albin RL, Hu H, Rozek LS (2012) Genome-wide DNA methylation differences between late-onset Alzheimer's disease and cognitively normal controls in human frontal cortex. *Journal of Alzheimer's disease : JAD* 29 (3):571-588. doi:10.3233/JAD-2012-111223

36. De Jager PL, Srivastava G, Lunnon K, Burgess J, Schalkwyk LC, Yu L, Eaton ML, Keenan BT, Ernst J, McCabe C, Tang A, Raj T, Replogle J, Brodeur W, Gabriel S, Chai HS, Younkin C, Younkin SG, Zou F, Szyf M, Epstein CB, Schneider JA, Bernstein BE, Meissner A, Ertekin-Taner N, Chibnik LB, Kellis M, Mill J, Bennett DA (2014) Alzheimer's disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDF2 and other loci. *Nature neuroscience* 17 (9):1156-1163. doi:10.1038/nn.3786

<http://www.nature.com/neuro/journal/v17/n9/abs/nn.3786.html#supplementary-information>

37. Lunnon K, Smith R, Hannon E, De Jager PL, Srivastava G, Volta M, Troakes C, Al-Sarraj S, Burrage J, Macdonald R, Condliffe D, Harries LW, Katsel P, Haroutunian V, Kaminsky Z, Joachim C, Powell J, Lovestone S, Bennett DA, Schalkwyk LC, Mill J (2014) Methylomic profiling implicates cortical deregulation of ANK1 in Alzheimer's disease. *Nature neuroscience* 17 (9):1164-1170. doi:10.1038/nn.3782

<http://www.nature.com/neuro/journal/v17/n9/abs/nn.3782.html#supplementary-information>

38. Watson CT, Roussos P, Garg P, Ho DJ, Azam N, Katsel PL, Haroutunian V, Sharp AJ (2016) Genome-wide DNA methylation profiling in the superior temporal gyrus reveals epigenetic signatures associated with Alzheimer's disease. *Genome Medicine* 8 (1):1-14

39. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, Fuks F (2011) Evaluation of the Infinium Methylation 450K technology. *Epigenomics* 3 (6):771-784. doi:10.2217/epi.11.105
40. Lord J, Cruchaga C (2014) The epigenetic landscape of Alzheimer's disease. *Nature neuroscience* 17 (9):1138-1140
41. Moran S, Arribas C, Esteller M (2016) Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics* 8 (3):389-399. doi:10.2217/epi.15.114
42. Busche S, Shao X, Caron M, Kwan T, Allum F, Cheung WA, Ge B, Westfall S, Simon MM, Multiple Tissue Human Expression R, Barrett A, Bell JT, McCarthy MI, Deloukas P, Blanchette M, Bourque G, Spector TD, Lathrop M, Pastinen T, Grundberg E (2015) Population whole-genome bisulfite sequencing across two tissues highlights the environment as the principal source of human methylome variation. *Genome biology* 16 (1):290. doi:10.1186/s13059-015-0856-1
43. Veillard A-C, Datlinger P, Laczik M, Squazzo S, Bock C (2016) Diagenode[reg] Premium RRBS technology: cost-effective DNA methylation mapping with superior coverage. *Nat Meth* 13 (2). doi:10.1038/nmeth.f.391
44. Booth MJ, Ost TW, Beraldi D, Bell NM, Branco MR, Reik W (2013) Oxidative bisulfite sequencing of 5-methylcytosine and 5-hydroxymethylcytosine. *Nat Protoc* 8. doi:10.1038/nprot.2013.115
45. Booth MJ, Marsico G, Bachman M, Beraldi D, Balasubramanian S (2014) Quantitative sequencing of 5-formylcytosine in DNA at single-base resolution. *Nature chemistry* 6 (5):435-440. doi:10.1038/nchem.1893
46. Booth MJ, Ost TW, Beraldi D, Bell NM, Branco MR, Reik W, Balasubramanian S (2013) Oxidative bisulfite sequencing of 5-methylcytosine and 5-hydroxymethylcytosine. *Nature protocols* 8 (10):1841-1851. doi:10.1038/nprot.2013.115
47. Iurlaro M, McInroy GR, Burgess HE, Dean W, Raiber EA, Bachman M, Beraldi D, Balasubramanian S, Reik W (2016) In vivo genome-wide profiling reveals a tissue-specific role for 5-formylcytosine. *Genome biology* 17 (1):141. doi:10.1186/s13059-016-1001-5
48. Stewart SK, Morris TJ, Guilhamon P, Bulstrode H, Bachman M, Balasubramanian S, Beck S (2015) oxBS-450K: A method for analysing hydroxymethylation using 450K BeadChips. *Methods* 72:9-15. doi:http://dx.doi.org/10.1016/j.jymeth.2014.08.009

49. Lunnon K, Hannon E, Smith RG, Dempster E, Wong C, Burrage J, Troakes C, Al-Sarraj S, Kepa A, Schalkwyk L, Mill J (2016) Variation in 5-hydroxymethylcytosine across human cortex and cerebellum. *Genome biology* 17 (1):1-15. doi:10.1186/s13059-016-0871-x
50. Coppieters N, Dieriks BV, Lill C, Faull RL, Curtis MA, Dragunow M (2014) Global changes in DNA methylation and hydroxymethylation in Alzheimer's disease human brain. *Neurobiology of aging* 35 (6):1334-1344
51. Condliffe D, Wong A, Troakes C, Proitsi P, Patel Y, Chouliaras L, Fernandes C, Cooper J, Lovestone S, Schalkwyk L, Mill J, Lunnon K (2014) Cross-region reduction in 5-hydroxymethylcytosine in Alzheimer's disease brain. *Neurobiology of aging* 35 (8):1850-1854. doi:10.1016/j.neurobiolaging.2014.02.002
52. Lashley T, Gami P, Valizadeh N, Li A, Revesz T, Balazs R (2015) Alterations in global DNA methylation and hydroxymethylation are not detected in Alzheimer's disease. *Neuropathol Appl Neurobiol* 41 (4):497-506. doi:10.1111/nan.12183
53. Chouliaras L, Mastroeni D, Delvaux E, Grover A, Kenis G, Hof PR, Steinbusch HW, Coleman PD, Rutten BP, van den Hove DL (2013) Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. *Neurobiology of aging* 34 (9):2091-2099
54. van den Hove DL, Chouliaras L, Rutten BP (2012) The role of 5-hydroxymethylcytosine in aging and Alzheimer's disease: current status and prospects for future studies. *Curr Alzheimer Res* 9 (5):545-549
55. Bradley-Whitman MA, Lovell MA (2013) Epigenetic changes in the progression of Alzheimer's disease. *Mechanisms of ageing and development* 134 (10):486-495. doi:http://dx.doi.org/10.1016/j.mad.2013.08.005
56. Zhang K, Schrag M, Crofton A, Trivedi R, Vinters H, Kirsch W (2012) Targeted proteomics for quantification of histone acetylation in Alzheimer's disease. *Proteomics* 12 (8):1261-1268
57. Mastroeni D, Delvaux E, Nolz J, Tan Y, Grover A, Oddo S, Coleman PD (2015) Aberrant intracellular localization of H3k4me3 demonstrates an early epigenetic phenomenon in Alzheimer's disease. *Neurobiology of aging* 36 (12):3121-3129. doi:http://dx.doi.org/10.1016/j.neurobiolaging.2015.08.017
58. Gräff J, Rei D, Guan J-S, Wang W-Y, Seo J, Hennig KM, Nieland TJ, Fass DM, Kao PF, Kahn M (2012) An epigenetic blockade of cognitive functions in the neurodegenerating brain. *Nature* 483 (7388):222-226

59. Zhou X, Xu J (2015) Identification of Alzheimer's disease-associated long noncoding RNAs. *Neurobiology of aging* 36 (11):2925-2931. doi:<http://dx.doi.org/10.1016/j.neurobiolaging.2015.07.015>
60. Deal RB, Henikoff S (2011) The INTACT method for cell type-specific gene expression and chromatin profiling in *Arabidopsis thaliana*. *Nature protocols* 6 (1):56-68. doi:10.1038/nprot.2010.175
61. Amin NM, Greco TM, Kuchenbrod LM, Rigney MM, Chung MI, Wallingford JB, Cristea IM, Conlon FL (2014) Proteomic profiling of cardiac tissue by isolation of nuclei tagged in specific cell types (INTACT). *Development* 141 (4):962-973. doi:10.1242/dev.098327
62. Nijholt DA, Stingl C, Luiders TM (2015) Laser capture microdissection of fluorescently labeled amyloid plaques from Alzheimer's disease brain tissue for mass spectrometric analysis. *Methods in molecular biology* 1243:165-173. doi:10.1007/978-1-4939-1872-0_9
63. Siniard AL, Corneveaux JJ, Both M, Chawla MK, Barnes CA, Huentelman MJ (2015) RNA Sequencing from Laser Capture Microdissected Brain Tissue to Study Normal Aging and Alzheimer's Disease. In: Jain KK (ed) *Applied Neurogenomics*. Springer New York, New York, NY, pp 111-120. doi:10.1007/978-1-4939-2247-5_4
64. Guintivano J, Aryee MJ, Kaminsky ZA (2013) A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. *Epigenetics* 8 (3):290-302. doi:10.4161/epi.23924
65. Sanchez-Mut JV, Heyn H, Vidal E, Moran S, Sayols S, Delgado-Morales R, Schultz MD, Ansoleaga B, Garcia-Esparcia P, Pons-Espinal M, de Lagran MM, Dopazo J, Rabano A, Avila J, Dierssen M, Lott I, Ferrer I, Ecker JR, Esteller M (2016) Human DNA methylomes of neurodegenerative diseases show common epigenomic patterns. *Translational psychiatry* 6:e718. doi:10.1038/tp.2015.214
66. Di Francesco A, Arosio B, Falconi A, Micioni Di Bonaventura MV, Karimi M, Mari D, Casati M, Maccarrone M, D'Addario C (2015) Global changes in DNA methylation in Alzheimer's disease peripheral blood mononuclear cells. *Brain, Behavior, and Immunity* 45:139-144. doi:<http://dx.doi.org/10.1016/j.bbi.2014.11.002>
67. Lahiri DK, Maloney B, Zawia NH (2009) The LEARN model: an epigenetic explanation for idiopathic neurobiological diseases. *Mol Psychiatry* 14 (11):992-1003. doi:<http://www.nature.com/mp/journal/v14/n11/supinfo/mp200982s1.html>

68. Lahiri DK, Maloney B (2010) The "LEARn" (Latent Early-life Associated Regulation) model integrates environmental risk factors and the developmental basis of Alzheimer's disease, and proposes remedial steps. *Experimental gerontology* 45 (4):291-296. doi:10.1016/j.exger.2010.01.001
69. Velez JI, Lopera F, Sepulveda-Falla D, Patel HR, Johar AS, Chuah A, Tobon C, Rivera D, Villegas A, Cai Y, Peng K, Arkell R, Castellanos FX, Andrews SJ, Silva Lara MF, Creagh PK, Easteal S, de Leon J, Wong ML, Licinio J, Mastronardi CA, Arcos-Burgos M (2015) APOE[ast]E2 allele delays age of onset in PSEN1 E280A Alzheimer's disease. *Mol Psychiatry*. doi:10.1038/mp.2015.177
70. Yang J, Yu L, Gaiteri C, Srivastava GP, Chibnik LB, Leurgans SE, Schneider JA, Meissner A, De Jager PL, Bennett DA (2015) Association of DNA methylation in the brain with age in older persons is confounded by common neuropathologies. *The International Journal of Biochemistry & Cell Biology* 67:58-64. doi:http://dx.doi.org/10.1016/j.biocel.2015.05.009
71. Smith AR, Mill J, Smith RG, Lunnon K (2016) Elucidating novel dysfunctional pathways in Alzheimer's disease by integrating loci identified in genetic and epigenetic studies. *Neuroepigenetics* 6:32-50. doi:http://dx.doi.org/10.1016/j.nepig.2016.05.001
72. Wagner JR, Busche S, Ge B, Kwan T, Pastinen T, Blanchette M (2014) The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. *Genome biology* 15 (2):R37. doi:10.1186/gb-2014-15-2-r37
73. Relton CL, Davey Smith G (2015) Mendelian randomization: applications and limitations in epigenetic studies. *Epigenomics* 7 (8):1239-1243. doi:10.2217/epi.15.88
74. Hannon E, Spiers H, Viana J, Pidsley R, Burrage J, Murphy TM, Troakes C, Turecki G, O'Donovan MC, Schalkwyk LC, Bray NJ, Mill J (2016) Methylation QTLs in the developing brain and their enrichment in schizophrenia risk loci. *Nature neuroscience* 19 (1):48-54. doi:10.1038/nn.4182
<http://www.nature.com/neuro/journal/v19/n1/abs/nn.4182.html#supplementary-information>
75. Lahiri DK, Maloney B (2012) Gene x environment interaction by a longitudinal epigenome-wide association study (LEWAS) overcomes limitations of genome-wide association study (GWAS). *Epigenomics* 4 (6):685-699. doi:10.2217/epi.12.60

76. Maloney B, Lahiri DK (2016) Epigenetics of dementia: understanding the disease as a transformation rather than a state. *The Lancet Neurology* 15 (7):760-774. doi:http://dx.doi.org/10.1016/S1474-4422(16)00065-X
77. Swerdlow RH (2007) Is aging part of Alzheimer's disease, or is Alzheimer's disease part of aging? *Neurobiology of aging* 28 (10):1465-1480. doi:10.1016/j.neurobiolaging.2006.06.021
78. Arnold SE, Lee EB, Moberg PJ, Stutzbach L, Kazi H, Han LY, Lee VM, Trojanowski JQ (2010) Olfactory epithelium amyloid-beta and paired helical filament-tau pathology in Alzheimer disease. *Annals of neurology* 67 (4):462-469. doi:10.1002/ana.21910
79. Dugger BN, Hidalgo JA, Chiarolanza G, Mariner M, Henry-Watson J, Sue LI, Beach TG (2013) The distribution of phosphorylated tau in spinal cords of Alzheimer's disease and non-demented individuals. *Journal of Alzheimer's disease : JAD* 34 (2):529-536. doi:10.3233/JAD-121864
80. Curcio CA, Kemper T (1984) Nucleus raphe dorsalis in dementia of the Alzheimer type: neurofibrillary changes and neuronal packing density. *J Neuropathol Exp Neurol* 43 (4):359-368
81. German DC, White CL, 3rd, Sparkman DR (1987) Alzheimer's disease: neurofibrillary tangles in nuclei that project to the cerebral cortex. *Neuroscience* 21 (2):305-312
82. Aston-Jones G, Cohen JD (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annual review of neuroscience* 28:403-450. doi:10.1146/annurev.neuro.28.061604.135709
83. Dringenberg HC (2000) Alzheimer's disease: more than a 'cholinergic disorder' - evidence that cholinergic-monoaminergic interactions contribute to EEG slowing and dementia. *Behavioural brain research* 115 (2):235-249
84. Grinberg LT, Rub U, Ferretti REL, Nitrini R, Farfel JM, Leite REP, Polichiso L, Alho ATL, Oliveira KC, Lima MC, Moreira EG, Santos EB, Andrade MP, Pasqualucci CA, Jacob-Filho W, Saldiva PHN, Gierga K, Heinsen H, Brazilian Brain Bank Study G (2008) The dorsal raphe nucleus shows phospho-tau cytoskeletal changes before the transentorhinal region in AD. A new (or earlier) beginning? *Acta Neuropathol* 116 (3):343-343
85. O'Donnell J, Zeppenfeld D, McConnell E, Pena S, Nedergaard M (2012) Norepinephrine: a neuromodulator that boosts the function of multiple cell types to optimize CNS performance. *Neurochemical research* 37 (11):2496-2512. doi:10.1007/s11064-012-0818-x

86. Braak H, Del Tredici K (2012) Where, when, and in what form does sporadic Alzheimer's disease begin? *Current Opinion in Neurology* 25 (6):708-714. doi:10.1097/WCO.0b013e32835a3432
87. Simic G, Stanic G, Mladinov M, Jovanov-Milosevic N, Kostovic I, Hof PR (2009) Annotation - Does Alzheimer's disease begin in the brainstem? *Neuropathology and applied neurobiology* 35 (6):532-554. doi:10.1111/j.1365-2990.2009.01038.x
88. Arendt T, Stieler J, Holzer M (2015) Brain hypometabolism triggers PHF-like phosphorylation of tau, a major hallmark of Alzheimer's disease pathology. *Journal of neural transmission* 122 (4):531-539. doi:10.1007/s00702-014-1342-8
89. Chalermpananupap T, Kinkead B, Hu WT, Kummer MP, Hammerschmidt T, Heneka MT, Weinshenker D, Levey AI (2013) Targeting norepinephrine in mild cognitive impairment and Alzheimer's disease. *Alzheimer's research & therapy* 5 (2):21. doi:10.1186/alzrt175
90. Grudzien A, Shaw P, Weintraub S, Bigio E, Mash DC, Mesulam MM (2007) Locus coeruleus neurofibrillary degeneration in aging, mild cognitive impairment and early Alzheimer's disease. *Neurobiology of aging* 28 (3):327-335. doi:10.1016/j.neurobiolaging.2006.02.007
91. Haglund M, Sjoberck M, Englund E (2006) Locus ceruleus degeneration is ubiquitous in Alzheimer's disease: possible implications for diagnosis and treatment. *Neuropathology : official journal of the Japanese Society of Neuropathology* 26 (6):528-532
92. Lyness SA, Zarow C, Chui HC (2003) Neuron loss in key cholinergic and aminergic nuclei in Alzheimer disease: a meta-analysis. *Neurobiology of aging* 24 (1):1-23
93. Marcyniuk B, Mann DM, Yates PO (1986) The topography of cell loss from locus caeruleus in Alzheimer's disease. *J Neurol Sci* 76 (2-3):335-345
94. Marien MR, Colpaert FC, Rosenquist AC (2004) Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain research Brain research reviews* 45 (1):38-78. doi:10.1016/j.brainresrev.2004.02.002
95. Mesulam M, Shaw P, Mash D, Weintraub S (2004) Cholinergic nucleus basalis tauopathy emerges early in the aging-MCI-AD continuum. *Annals of neurology* 55 (6):815-828. doi:10.1002/ana.20100

96. Trillo L, Das D, Hsieh W, Medina B, Moghadam S, Lin B, Dang V, Sanchez MM, De Miguel Z, Ashford JW, Salehi A (2013) Ascending monoaminergic systems alterations in Alzheimer's disease. translating basic science into clinical care. *Neuroscience and biobehavioral reviews* 37 (8):1363-1379. doi:10.1016/j.neubiorev.2013.05.008
97. Weinshenker D (2008) Functional consequences of locus coeruleus degeneration in Alzheimer's disease. *Curr Alzheimer Res* 5 (3):342-345
98. Zweig RM, Ross CA, Hedreen JC, Steele C, Cardillo JE, Whitehouse PJ, Folstein MF, Price DL (1989) Neuropathology of aminergic nuclei in Alzheimer's disease. *Progress in clinical and biological research* 317:353-365
99. Busch C, Bohl J, Ohm TG (1997) Spatial, temporal and numeric analysis of Alzheimer changes in the nucleus coeruleus. *Neurobiology of aging* 18 (4):401-406
100. Parvizi J, Van Hoesen GW, Damasio A (2001) The selective vulnerability of brainstem nuclei to Alzheimer's disease. *Annals of neurology* 49 (1):53-66
101. Sara SJ (2009) The locus coeruleus and noradrenergic modulation of cognition. *Nat Rev Neurosci* 10 (3):211-223. doi:10.1038/nrn2573
102. Schliebs R, Arendt T (2011) The cholinergic system in aging and neuronal degeneration. *Behavioural brain research* 221 (2):555-563. doi:10.1016/j.bbr.2010.11.058
103. Theofilas P, Dunlop S, Heinsen H, Grinberg LT (2015) Turning on the Light Within: Subcortical Nuclei of the Isodentritic Core and their Role in Alzheimer's Disease Pathogenesis. *Journal of Alzheimers Disease* 46 (1):17-34. doi:10.3233/jad-142682
104. Rub U, Del Tredici K, Schultz C, Thal DR, Braak E, Braak H (2000) The evolution of Alzheimer's disease-related cytoskeletal pathology in the human raphe nuclei. *Neuropathol Appl Neurobiol* 26 (6):553-567
105. Arendt T, Bruckner MK, Morawski M, Jager C, Gertz HJ (2015) Early neurone loss in Alzheimer's disease: cortical or subcortical? *Acta neuropathologica communications* 3:10. doi:10.1186/s40478-015-0187-1
106. Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Thies B, Trojanowski JQ, Vinters HV, Montine TJ (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. *Alzheimer's & Dementia* 8 (1):1-13. doi:http://dx.doi.org/10.1016/j.jalz.2011.10.007

107. Braak H, Braak E (1997) Frequency of stages of Alzheimer-related lesions in different age categories. *Neurobiology of aging* 18 (4):351-357
108. Lee JH, Ryan J, Andreescu C, Aizenstein H, Lim HK (2015) Brainstem morphological changes in Alzheimer's disease. *Neuroreport* 26 (7):411-415. doi:10.1097/wnr.0000000000000362
109. Guo X, Waern M, Sjogren K, Lissner L, Bengtsson C, Bjorkelund C, Ostling S, Gustafson D, Skoog I (2007) Midlife respiratory function and Incidence of Alzheimer's disease: a 29-year longitudinal study in women. *Neurobiology of aging* 28 (3):343-350. doi:10.1016/j.neurobiolaging.2006.01.008
110. Grinberg LT, Rub U, Ferretti REL, Nitrini R, Farfel JM, Polichiso L, Gierga K, Jacob W, Heinsen H, Brazilian Brain Bank Study G (2009) The dorsal raphe nucleus shows phospho-tau neurofibrillary changes before the transentorhinal region in Alzheimer's disease. A precocious onset? *Neuropathology and Applied Neurobiology* 35 (4):406-416. doi:10.1111/j.1365-2990.2008.00997.x
111. Ramos-Rodriguez JJ, Molina-Gil S, Rey-Brea R, Berrocoso E, Garcia-Alloza M (2013) Specific Serotonergic Denervation Affects tau Pathology and Cognition without Altering Senile Plaques Deposition in APP/PS1 Mice. *PLoS ONE* 8 (11):e79947. doi:10.1371/journal.pone.0079947
112. Mann DM, Lincoln J, Yates PO, Stamp JE, Toper S (1980) Changes in the monoamine containing neurones of the human CNS in senile dementia. *The British journal of psychiatry : the journal of mental science* 136:533-541
113. Tomlinson BE, Irving D, Blessed G (1981) Cell loss in the locus coeruleus in senile dementia of Alzheimer type. *J Neurol Sci* 49 (3):419-428
114. Marcyniuk B, Mann DM, Yates PO (1986) Loss of nerve cells from locus coeruleus in Alzheimer's disease is topographically arranged. *Neuroscience letters* 64 (3):247-252
115. Morsch R, Simon W, Coleman PD (1999) Neurons may live for decades with neurofibrillary tangles. *J Neuropathol Exp Neurol* 58 (2):188-197
116. Frazer A HJ (1999) Serotonin Involvement in Physiological Function and Behavior. *Basic Neurochemistry: Molecular, Cellular and Medical Aspects*, 6th edn., Philadelphia: Lippincott-Raven
117. Baker KG, Halliday GM, Tork I (1990) Cytoarchitecture of the human dorsal raphe nucleus. *The Journal of comparative neurology* 301 (2):147-161. doi:10.1002/cne.903010202

118. Hornung JP (2003) The human raphe nuclei and the serotonergic system. *Journal of chemical neuroanatomy* 26 (4):331-343
119. Grinberg LT, Rub U, Ferretti RE, Nitrini R, Farfel JM, Polichiso L, Gierga K, Jacob-Filho W, Heinsen H, Brazilian Brain Bank Study G (2009) The dorsal raphe nucleus shows phospho-tau neurofibrillary changes before the transentorhinal region in Alzheimer's disease. A precocious onset? *Neuropathol Appl Neurobiol* 35 (4):406-416. doi:10.1111/j.1365-2990.2009.00997.x
120. Mirbaha H, Holmes BB, Sanders DW, Bieschke J, Diamond MI (2015) Tau Trimers Are the Minimal Propagation Unit Spontaneously Internalized to Seed Intracellular Aggregation. *The Journal of biological chemistry* 290 (24):14893-14903. doi:10.1074/jbc.M115.652693
121. Rodriguez JJ, Noristani HN, Verkhatsky A (2012) The serotonergic system in ageing and Alzheimer's disease. *Prog Neurobiol* 99 (1):15-41. doi:10.1016/j.pneurobio.2012.06.010
122. Lai MK, Tsang SW, Francis PT, Keene J, Hope T, Esiri MM, Spence I, Chen CP (2002) Postmortem serotonergic correlates of cognitive decline in Alzheimer's disease. *Neuroreport* 13 (9):1175-1178
123. Chen CP, Eastwood SL, Hope T, McDonald B, Francis PT, Esiri MM (2000) Immunocytochemical study of the dorsal and median raphe nuclei in patients with Alzheimer's disease prospectively assessed for behavioural changes. *Neuropathol Appl Neurobiol* 26 (4):347-355
124. Wu Y-H, Feenstra MGP, Zhou J-N, Liu R-Y, Toranö JS, Kan HJMV, Fischer DF, Ravid R, Swaab DF (2003) Molecular Changes Underlying Reduced Pineal Melatonin Levels in Alzheimer Disease: Alterations in Preclinical and Clinical Stages. *The Journal of Clinical Endocrinology & Metabolism* 88 (12):5898-5906. doi:doi:10.1210/jc.2003-030833
125. Kepe V, Barrio JR, Huang S-C, Ercoli L, Siddarth P, Shoghi-Jadid K, Cole GM, Satyamurthy N, Cummings JL, Small GW, Phelps ME (2006) Serotonin 1A receptors in the living brain of Alzheimer's disease patients. *Proceedings of the National Academy of Sciences of the United States of America* 103 (3):702-707. doi:10.1073/pnas.0510237103
126. Engelborghs S, Sleegers K, Van der Mussele S, Le Bastard N, Brouwers N, Van Broeckhoven C, De Deyn PP (2013) Brain-specific tryptophan hydroxylase, TPH2, and 5-HTTLPR are associated with frontal lobe symptoms in Alzheimer's disease. *Journal of Alzheimer's disease : JAD* 35 (1):67-73. doi:10.3233/JAD-101305

127. Cirrito JR, Disabato BM, Restivo JL, Verges DK, Goebel WD, Sathyan A, Hayreh D, D'Angelo G, Benzinger T, Yoon H, Kim J, Morris JC, Mintun MA, Sheline YI (2011) Serotonin signaling is associated with lower amyloid- β levels and plaques in transgenic mice and humans. *Proceedings of the National Academy of Sciences* 108 (36):14968-14973. doi:10.1073/pnas.1107411108
128. Sheline YI, West T, Yarasheski K, Swarm R, Jasielec MS, Fisher JR, Ficker WD, Yan P, Xiong C, Frederiksen C, Grzelak MV, Chott R, Bateman RJ, Morris JC, Mintun MA, Lee J-M, Cirrito JR (2014) An Antidepressant Decreases CSF A β Production in Healthy Individuals and in Transgenic AD Mice. *Science Translational Medicine* 6 (236):236re234-236re234. doi:10.1126/scitranslmed.3008169
129. Salloway S, Sperling R, Fox NC, Blennow K, Klunk W, Raskind M, Sabbagh M, Honig LS, Porsteinsson AP, Ferris S, Reichert M, Ketter N, Nejadnik B, Guenzler V, Miloslavsky M, Wang D, Lu Y, Lull J, Tudor IC, Liu E, Grundman M, Yuen E, Black R, Brashear HR, Bapineuzumab, Clinical Trial I (2014) Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *The New England journal of medicine* 370 (4):322-333. doi:10.1056/NEJMoa1304839
130. Geldenhuys WJ, Van der Schyf CJ (2011) Role of serotonin in Alzheimer's disease: a new therapeutic target? *CNS drugs* 25 (9):765-781. doi:10.2165/11590190-000000000-00000
131. Robert SJ, Zugaza JL, Fischmeister R, Gardier AM, Lezoualc'h F (2001) The human serotonin 5-HT₄ receptor regulates secretion of non-amyloidogenic precursor protein. *The Journal of biological chemistry* 276 (48):44881-44888. doi:10.1074/jbc.M109008200
132. Benarroch EE (2009) The locus ceruleus norepinephrine system. *Neurology* 73 (20):1699-1704. doi:http://dx.doi.org/10.1212/WNL.0b013e3181c2937c
133. Tejani-Butt SM, Yang J, Zaffar H (1993) Norepinephrine transporter sites are decreased in the locus coeruleus in Alzheimer's disease. *Brain Res* 631 (1):147-150
134. Chan-Palay V, Asan E (1989) Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. *The Journal of comparative neurology* 287 (3):373-392. doi:10.1002/cne.902870308
135. Wilson RS, Boyle PA, Yu L, Barnes LL, Schneider JA, Bennett DA (2013) Life-span cognitive activity, neuropathologic burden, and cognitive aging. *Neurology* 81 (4):314-321. doi:10.1212/WNL.0b013e31829c5e8a

136. Szot P, White SS, Greenup JL, Leverenz JB, Peskind ER, Raskind MA (2006) Compensatory changes in the noradrenergic nervous system in the locus ceruleus and hippocampus of postmortem subjects with Alzheimer's disease and dementia with Lewy bodies. *The Journal of neuroscience* 26 (2):467-478
137. Hoogendijk WJ, Feenstra MG, Botterblom MH, Gilhuis J, Sommer IE, Kamphorst W, Eikelenboom P, Swaab DF (1999) Increased activity of surviving locus ceruleus neurons in Alzheimer's disease. *Annals of neurology* 45 (1):82-91
138. Storga D, Vrecko K, Birkmayer JG, Reibnegger G (1996) Monoaminergic neurotransmitters, their precursors and metabolites in brains of Alzheimer patients. *Neuroscience letters* 203 (1):29-32
139. Palmer AM, Francis PT, Bowen DM, Benton JS, Neary D, Mann DM, Snowden JS (1987) Catecholaminergic neurones assessed ante-mortem in Alzheimer's disease. *Brain Res* 414 (2):365-375
140. Vermeiren Y, Van Dam D, Aerts T, Engelborghs S, De Deyn PP (2014) Monoaminergic neurotransmitter alterations in postmortem brain regions of depressed and aggressive patients with Alzheimer's disease. *Neurobiology of aging* 35 (12):2691-2700
141. Herrmann N, Lanctot KL, Eryavec G, Khan LR (2004) Noradrenergic activity is associated with response to pindolol in aggressive Alzheimer's disease patients. *Journal of psychopharmacology* 18 (2):215-220. doi:10.1177/0269881104042625
142. Raskind MA, Peskind ER, Halter JB, Jimerson DC (1984) Norepinephrine and MHPG levels in CSF and plasma in Alzheimer's disease. *Archives of general psychiatry* 41 (4):343-346
143. Tohgi H, Ueno M, Abe T, Takahashi S, Nozaki Y (1992) Concentration of monoamines and their metabolites in the cerebrospinal fluid from patients with senile dementia of the Alzheimer type and vascular dementia of the Binswanger type. *Journal of Neural Transmission: Parkinson's Disease and Dementia Section* 4 (1):69-77
144. Kopin IJ, Gordon E, Jimerson D, Polinsky R (1983) Relation between plasma and cerebrospinal fluid levels of 3-methoxy-4-hydroxyphenylglycol. *Science* 219 (4580):73-75
145. Cristy P, Atoossa F, Devsmita D, Fatemeh SM, Ravikumar P, Ahmad S (2016) Noradrenergic System in Down Syndrome and Alzheimer's Disease A Target for Therapy. *Current Alzheimer Research* 13 (1):68-83. doi:http://dx.doi.org/10.21

74/1567205012666150921095924

146. Cross AJ, Crow TJ, Perry EK, Perry RH, Blessed G, Tomlinson BE (1981) Reduced dopamine-beta-hydroxylase activity in Alzheimer's disease. *British Medical Journal (Clinical research ed)* 282 (6258):93-94

147. Mustapic M, Presecki P, Pivac N, Mimica N, Hof PR, Simic G, Folnegovic-Smalc V, Muck-Seler D (2013) Genotype-independent decrease in plasma dopamine beta-hydroxylase activity in Alzheimer's disease. *Progress in neuro-psychopharmacology & biological psychiatry* 44:94-99. doi:10.1016/j.pnpbp.2013.02.002

148. Segal SK, Cotman CW, Cahill LF (2012) Exercise-induced noradrenergic activation enhances memory consolidation in both normal aging and patients with amnesic mild cognitive impairment. *Journal of Alzheimer's disease : JAD* 32 (4):1011-1018. doi:10.3233/JAD-2012-121078

149. Kalinin S, Gavriluk V, Polak PE, Vasser R, Zhao J, Heneka MT, Feinstein DL (2007) Noradrenaline deficiency in brain increases beta-amyloid plaque burden in an animal model of Alzheimer's disease. *Neurobiology of aging* 28 (8):1206-1214. doi:10.1016/j.neurobiolaging.2006.06.003

150. Kalinin S, Polak PE, Lin SX, Sakharkar AJ, Pandey SC, Feinstein DL (2012) The noradrenaline precursor L-DOPS reduces pathology in a mouse model of Alzheimer's disease. *Neurobiology of aging* 33 (8):1651-1663. doi:10.1016/j.neurobiolaging.2011.04.012

151. Mehler MF, Purpura DP (2009) Autism, fever, epigenetics and the locus coeruleus. *Brain research reviews* 59 (2):388-392. doi:10.1016/j.brainresrev.2008.11.001

152. Schanen NC (2006) Epigenetics of autism spectrum disorders. *Human molecular genetics* 15 Spec No 2:R138-150. doi:10.1093/hmg/ddl213

153. Homberg JR, van den Hove DL (2012) The serotonin transporter gene and functional and pathological adaptation to environmental variation across the life span. *Prog Neurobiol* 99 (2):117-127. doi:10.1016/j.pneurobio.2012.08.003

154. Schraut KG, Jakob SB, Weidner MT, Schmitt AG, Scholz CJ, Strekalova T, El Hajj N, Eijssen LM, Domschke K, Reif A, Haaf T, Ortega G, Steinbusch HW, Lesch KP, Van den Hove DL (2014) Prenatal stress-induced programming of genome-wide promoter DNA methylation in 5-HTT-deficient mice. *Translational psychiatry* 4:e473. doi:10.1038/tp.2014.107

155. Waltes R, Chiocchetti AG, Freitag CM (2015) The neurobiological basis of human aggression: A review on genetic and epigenetic mechanisms. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*:n/a-n/a. doi:10.1002/ajmg.b.32388
156. Van den Hove D, Jakob SB, Schraut K-G, Kenis G, Schmitt AG, Kneitz S, Scholz C-J, Wiescholleck V, Ortega G, Prickaerts J, Steinbusch H, Lesch K-P (2011) Differential Effects of Prenatal Stress in 5-Htt Deficient Mice: Towards Molecular Mechanisms of Gene × Environment Interactions. *PLoS ONE* 6 (8):e22715. doi:10.1371/journal.pone.0022715
157. Guidotti A, Auta J, Davis JM, Dong E, Gavin DP, Grayson DR, Sharma RP, Smith RC, Tueting P, Zhubi A (2014) Toward the identification of peripheral epigenetic biomarkers of schizophrenia. *Journal of neurogenetics* 28 (1-2):41-52. doi:10.3109/01677063.2014.892485
158. Sinicrope FA, Okamoto K, Kasi PM, Kawakami H (2016) Molecular Biomarkers in the Personalized Treatment of Colorectal Cancer. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. doi:10.1016/j.cgh.2016.02.008
159. Cherradi N (2015) microRNAs as Potential Biomarkers in Adrenocortical Cancer: Progress and Challenges. *Frontiers in endocrinology* 6:195. doi:10.3389/fendo.2015.00195
160. Huang TL, Lin CC (2015) Advances in biomarkers of major depressive disorder. *Advances in clinical chemistry* 68:177-204. doi:10.1016/bs.acc.2014.11.003
161. Ganesan A (2016) Multitarget Drugs: an Epigenetic Epiphany. *ChemMedChem*:n/a-n/a. doi:10.1002/cmdc.201500394

Methylomic and hydroxymethylomic profiling of the dorsal raphe nucleus in Alzheimer's disease

Abstract	p.76
Keywords	p.76
Introduction	p.77
Methods	p.79
Sample Information and preparation	p.79
Bisulfite (BS) and oxidative-bisulfite (oxBS) treatment	p.79
Illumina Infinium MethylationEPIC BeadArray	p.79
Quality control (QC) and data normalization	p.80
Data analysis	p.80
DNA methylation and hydroxymethylation levels	p.80
Regression model	p.81
WGCNA and Gene ontology (GO)	p.81
Results	p.82
Differentially modified and unmodified cytosine profiles in the DRN	p.82
WGCNA and GO	p.83
Discussion	p.84
References	p.102

Artemis Iatrou¹, Ehsan Pishva^{1,2}, Roy Lardenoije¹, Janou Roubroeks², Joe Burrage², Bart Rutten¹, Jonathan Mill², Gunter Kenis¹, Katie Lunnon², Daniel L.A. van den Hove^{1,3}

1 School for Mental Health and Neuroscience (MHeNs), Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, the Netherlands

2 University of Exeter Medical School, University of Exeter, Exeter, UK

3 Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Wuerzburg, Germany

Abstract

Alzheimer's disease (AD) is a lethal neurodegenerative disease that affects brain integrity and functionality, resulting in progressive cognitive deterioration. And while cognitive impairment is the symptom most commonly associated with the clinical manifestation of the disease, early, preclinical symptomatology involves non-cognitive, neuropsychological features, functionally associated with brainstem structures. In the present study, the first of its kind, the dorsal raphe nucleus (DRN)-specific genome-wide patterns of methylated (5mC), hydroxymethylated (5hmC) as well as unmodified (UC) cytosines were investigated by means of the Illumina MethylationEPIC array, in relation to disease progression as characterized by Braak staging scores. The discrimination among the three methylation states was achieved by parallel treatment of the samples with bisulfite (BS) and oxidative bisulfite (oxBS), allowing for distinction of the true methylation signal from the global, methylation and hydroxymethylation one. Moreover, weighted gene co-expression analysis (WGCNA) was employed in order to investigate the association of co-methylated probes with disease progression in a systems-level and subsequent pathway analysis was performed for the identification of biological pathways differentially regulated with the aggravation of the pathology. Notably, some reported modified and unmodified genomic positions and regions have not been reported in epigenome-wide studies yet, but the genes affected have been previously shown to be involved in the pathophysiology of AD, specifically with respect to amyloid beta pathology. This study represents the first systematic epigenetic analysis, distinguishing among 5mC, 5hmC and UC in the DRN of AD patients making use of EPIC array technology. To this end, a unique structure-specific epigenomic profile in association with Braak stage progression was underlined.

Keywords: Alzheimer's disease; Brainstem; Dorsal raphe nucleus; Epigenetics; DNA methylation; DNA hydroxymethylation

Introduction

With the ever-increasing lifespan of the human population, 10 million new cases of Alzheimer's disease (AD) are reported annually [1], a number likely to multiply due to lack of effective therapeutic treatment options. Currently available treatment strategies only temporarily alleviate some symptoms but fail to suspend the deleterious underlying molecular and cellular events and thus are considered suboptimal. The disease is characterized by progressive accumulation of amyloid plaques and neurofibrillary tangles in the brain, which ultimately lead to neurodegeneration including widespread neuronal atrophy and cell death. The anatomical propagation of these hallmarks follows different patterns. Amyloid pathology exhibits a rather heterogeneous distribution and is weakly associated with the symptomatology of AD, hinting that it might trigger neurodegenerative cascades independently of its spreading [2]. On the other hand, tau pathology propagates in an orchestrated and predictable ascending pattern, from subcortical to cortical structures, and seems to be rather selective to specific neuronal networks [3, 4]. Based on the latter observation, Braak and Braak introduced a 7-point system for staging AD representing the spreading pattern of tau pathology [5]. The conveyance of the pathology to higher cortical areas marks the manifestation of clinical symptoms and the time point where therapeutic treatment is provided. However, at that relatively advanced stage, the diseased brain appears to be unresponsive to therapy [6]. Strikingly, the first reported signs of tau pathology appear as early as the fourth decade of life, within the brainstem, more specifically in the magnocellular nuclei of the basal forebrain, the raphe nuclei and the locus coeruleus (LC), structures highly overlooked in present AD research lines [7-10]. This observation substantiates the preclinical symptomatology that involves non-cognitive, behavioral and neuropsychological phenotypes, such as depression, general disturbances in mood, emotion, appetite, respiration and circadian rhythm [3]. Understanding the pathophysiology of AD in these brain areas is imperative for the development of an effective treatment and for elucidating the rather enigmatic facet of the onset of AD would facilitate scientific breakthroughs in diagnostics and therapeutics.

To date, the long-prevailing theories of genetic contribution to AD susceptibility do not adequately explain the pathogenesis of the disease, regardless the high heritability estimates (58-79%)[11]. Outcomes from genome-wide association studies (GWAS) have indicated multiple genetic variants associated with an elevated risk of developing AD, yet these identified single nucleotide polymorphisms (SNPs) are reported to account only for 24-33% of attributable risk in AD [12]. Thus, while genetic factors clearly play a role in the pathogenesis of AD, other, non-genetic factors are of utter importance for the initiation and progression of the disease. To this end, epigenome-wide association studies (EWAS) have been employed in order to identify epigenetic changes in AD and examine the importance of epigenetic dysregulation in disease onset and progression, either in conjunction with or independently from genetic and environmental factors.

Epigenetic processes mediate reversible changes in gene expression via covalent interactions mainly impacting upon chromatin structure. Normal dynamic modifications in the epigenetic machinery are responsible for cellular development and differentiation, regulating the unique phenotype of each individual and additionally for transiently imprinting environmental, behavioral as well as social effects on gene expression, maintaining genomic homeostasis throughout the lifespan [13]. The best characterized epigenetic modification is DNA methylation, the addition of a methylation tag on a cytosine (C) at cytosine-phosphate-guanine (CpG) sites, i.e. regions of DNA where a C nucleotide is followed by a guanine (G) nucleotide in the linear sequence of bases, by DNA methyltransferases, forming 5-methylcytosine (5mC). Contrary to popular belief, DNA methylation is not solely associated with gene repression, but the differential effect on gene activity depends on the location of the epigenetic modification within the genetic locus [14]. The rapid development of improved epigenomic technologies has considerably assisted researchers to gain more insight in DNA demethylation variations. The more recently described demethylation intermediate, 5-hydroxymethylcytosine (5hmC), is speculated to have independent functional roles as it has been shown that 5hmC is enriched in the cerebellum and other brain regions, particularly in synapse-regulating genes [15]. To date, EWAS have identified AD-associated (hydroxy)methylation signatures in various loci, within the hippocampal formation and neighboring cortical areas, broadening the knowledge about the molecular mechanisms of AD [16, 17]. Yet, as these brain structures are particularly affected in more advanced stages of AD, epigenetic signatures identified therein, are more likely to reflect the progression (i.e. consequence), rather than the origin (i.e. cause) of this disorder. Therefore, the interrogation of the (hydroxy)methylome within brainstem nuclei could potentially provide important insight in how AD pathology starts and spreads.

Aiming to understand the role of epigenetic modifications within the brainstem and their impact on the development and progression of AD, we conducted a study using (oxidative) bisulfite-converted DNA combined with the use of Illumina MethylationEPIC arrays enabling us to distinguish between DNA methylation and hydroxymethylation interrogating the (hydroxyl)methylation status of more than 850,000 CpGs across the genome. We identified differentially unmodified and modified (methylated and hydroxymethylated) positions and regions in the dorsal raphe of AD patients over the progression of the disease. Consecutively, we examined the correlation of AD-associated (hydroxy)methylated positions throughout the genome and we unveiled biological pathways in which genes harboring these probes are involved. The technology used as well as the focus on the DRN makes this study highly innovative. By investigating (hydroxyl)methylation changes over Braak stages, this study attempted to model the epigenetic changes associated with AD progression in a timely manner and therefore would facilitate the translation towards more valid diagnostic and therapeutic discoveries.

Methods

Sample Information and preparation

Brain samples were obtained from the London Neurodegenerative Disease Brain Bank (LNDBB; <http://www.kcl.ac.uk/ioppn/depts/bcn/Our-research/Neurodegeneration/brain-bank.aspx>). Samples were provided with informed consent according to the Declaration of Helsinki (1991) and ethical approval for the study was provided by the NHS South East London REC 3. DRN tissue samples from 95 post-mortem brains from both AD patients and neurologically normal controls were provided. Demographic and relevant information about the samples are provided in Table 1. Subjects were distributed to the relevant Braak stage upon screening for AD neurofibrillary pathology. All samples were dissected by trained specialists, snap frozen, and stored at -80 °C. For each sample, genomic DNA was extracted from 50 mg of tissue using a standard phenol-chloroform extraction method.

Bisulfite (BS) and oxidative-bisulfite (oxBS) treatment

The TrueMethyl™ 24 Kit version 2.0 by CEGX™ (Cambridge Epigenetix Limited, Cambridge, UK) was used for bisulfite (BS) and oxidative bisulfite (oxBS) conversion of genomic DNA (gDNA). All laboratory procedures were performed at GenomeScan (GenomeScan B.V., Leiden, the Netherlands), according to the manufacturer's instructions. Prior to the conversion, high molecular weight genomic DNA (HMW gDNA) was quantified using a PicoGreen assay (Invitrogen, Carlsbad, California, USA), and gel-electrophoresis was performed to assess gDNA quality. All samples were of sufficient quantity and quality. A volume of 1 µg HMW gDNA was used per sample, which, after purification and denaturation, was split into two samples which underwent either DNA oxidation (oxBS samples) or mock DNA oxidation (BS samples). Subsequently, all samples were BS treated, and the yield of the samples was assessed by a Qubit ssDNA assay (Invitrogen). An additional restriction quality control was performed for a qualitative assessment of 5hmC oxidation and BS conversion.

Illumina Infinium MethylationEPIC BeadArray

From each BS/oxBS-treated DNA sample, 8 µL was amplified and hybridized on the Infinium MethylationEPIC BeadChip (EPIC array; Illumina, Inc., San Diego, CA, U.S.A.). All samples were randomized with respect to sex and disease status in order to avoid batch effects and were processed in batches of 24 BeadChips. BS and their matching oxBS samples were run on the same chip. Illumina iScan was used for imaging of the array. Sample preparation, hybridization, and washing steps for the Illumina Infinium II Methylation Assay of the BeadChip arrays were performed according to the manufacturer's protocol.

Quality control (QC) and data normalization

Illumina Genome Studio software was used to extract the raw signal intensities, visualized by Illumina iScanner. Further computations and statistical analyses were performed using R (version 3.3.2; <https://www.r-project.org>) and RStudio (version 1.0.153). The methylumi package was used in order to import the GenomeStudio reports into the R statistical environment [18]. Four samples were dropped out as they underperformed, according to the report provided by GenomeScan. Initial quality control assessment was performed using functions in the methylumi package to check concordance between reported and genotype-based gender in oxBS and BS samples. An additional quality control test was performed to filter out 6 samples displaying inefficient BS conversion ($< 75\%$). Further preprocessing was conducted using the watermelon package [19] by applying a p-filter, which removed 4 samples having more than 1% probes with a detection p-value greater than 0.05, in addition to removing 317 probes with a beadcount lower than 3 in more than 5% of samples, and 15098 probes having more than 1% of samples with a detection p-value greater than 0.05. Finally, in order to identify the most optimal normalization strategy from the watermelon package, 13 functions were assessed and ranked based on three performance metrics [19]. Dasen, which performed consistently well, was subsequently chosen for data normalization (Supplementary table 1). Non-CpG SNP probes, probes that have been reported to contain common ($MAF > 5\%$) SNPs in the CG or single base extension position, or probes that were non-specific or mismapped [20, 21], were flagged and disregarded in the evaluation of our results, leaving 92 BS-converted, 84 oxBS-converted samples and 778,408 probes for analysis. The distribution of beta (β) values from each dataset can be found in Supplementary figure 1.

Data analysis

DNA methylation and hydroxymethylation levels

For this study, we examined three cytosine states; unmodified C (UC), methylated C (5mC), and hydroxymethylated C (5hmC). DNA methylation levels were determined by the oxBS signal (β oxBS), whereas DNA hydroxymethylation levels of each sample were determined by subtracting the oxBS signal from the BS signal (β BS - β oxBS) for each probe in each sample. The remaining fraction ($1 - \beta$ BS) represented UC levels. While the oxBS treatment distinguishes true DNA methylation of cytosines from DNA hydroxymethylation, this method for defining hydroxymethylation does not exclude the co-existence of other DNA demethylation intermediates such as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Nevertheless, these oxidative bi-products are not enriched in the brain [22]. Due to technical variation, some 5hmC values were negative. In order to solely detect true hydroxymethylation, only 5hmC values of those probes displaying

significantly higher average BS when compared to oxBS levels, as assessed using a paired t-test, were used [23]. To this end, 329,671 probes survived the filtering for 5hmC.

Regression model

Differentially methylated, hydroxymethylated, and unmodified cytosine positions (DMPs, DHPs, DUPs) associated with disease progression were identified. A linear regression analysis was performed with the extracted 5mC, 5hmC and UC signals as outcome and Braak Stage as predictor (used as a continuous variable). Surrogate variable analysis (SVA) was performed in order to determine and estimate variation stemming from unknown sources, a common phenomenon in high-throughput experiments [24]. The identified surrogate variables (SVs) were then correlated with variables known to affect methylation status, i.e. age, gender, cell type composition, post mortem interval, and five SVs, the ones explaining most of the variation, were included as covariates in the model along with age and sex, factors known to influence methylation levels (Supplementary figure 2). The fit of the model generated was assessed based on the lambda (λ) value and the respective QQ plot (Supplementary figure 3). P-values were adjusted for multiple testing using the Benjamin-Hochberg, false discovery rate (FDR) procedure. Probes were ordered based on a combined p-value and regression estimate/coefficients ranking. Illumina EPIC array probes were annotated using the Illumina UCSC gene annotation.

Differentially (hydroxy)methylated and unmodified regions In order to identify differentially (hydroxy)methylated and unmodified regions (DHR, DMR, DUR), comb-p was used [25]. With this package, the p-values of DHP, DMP and DUP with nominal p-values lower than 0.001 were combined in sliding windows of 1000 base pairs and were spatially correlated across the genome. Obtained p-values were Stouffer-Liptak-Kechris (SLK) corrected for adjacent p-values, and were subsequently corrected for multiple testing using the Šidák correction.

WGCNA and Gene ontology (GO)

WGCNA was used to identify modules of highly co-methylated probes [26, 27]. Probes were filtered based on in (hydroxyl)methylation variation and the subset above the median was selected as input for further analysis ($n = 384,911$ probes for the 5mC dataset, $n = 164,835$ for the 5hmC dataset, $n = 389,204$ for the UC dataset). Modules were identified from their pairwise correlations, using a signed block-wise network construction at power 4 for the BS and 5hmC datasets and at power 5 for the 5mC (data not shown). Modules identified were assigned a colour name, and a weighted average-methylation profile, known as a module eigengene (ME), was calculated for each. Correlations between the MEs, Braak stage, phenotypic traits (age and sex) and surrogate variables

included in the regression model were used to identify modules associated with these variables. The relationship between each probe and each module was assessed through calculation of module membership (MM), the absolute correlation between a probe's DNA methylation status and the ME, allowing the identification of the subset of probes with the strongest membership to each module. Probe significance (PS), the absolute value of correlation between each probe and Braak stage, was calculated to quantify the association of the methylated probes with disease progression. For downstream explanation of the biological meaning of the modules more highly associated with Braak stage, genes associated with probes with an absolute MM value of ≥ 0.65 were selected. The gene annotation from Illumina was converted into ENTREZ-ID using the MissMethyl package [28] and gene ontology (GO) terms were downloaded and annotated to these probes.

Results

Differentially modified and unmodified cytosine profiles in the DRN

Firstly, DUPs, DMPs and DHPs associated with the progression of AD, as described by Braak stage, were identified (Table 2). Within the dataset of the unmodified cytosines, 5.9% of the probes (45,883) were significantly associated with Braak stage progression ($p \leq 0.05$). After FDR correction, 2 DUPs remained statistically significant, i.e. *IFFO2* (cg11237901, nominal $P = 1.47E-09$, $q = 0.0011$) and *PLCL2* (cg22927063, nominal $P = 1.16E-07$, $q = 0.043$) (Figure 1). Focusing on modified cytosines, methylation status of 5.2% of the probes (40,458) and hydroxymethylation status of 5.9% of the probes (19,272) was significantly associated with the progression of the disease. No DMPs or DHPs reached statistical significance after FDR correction. Detailed list of the most significant DUPs, DMPs and DHPs can be found in Table 2 and the respective Manhattan plots in Figure 2.

Subsequently, DURs, DMRs and DHRs were additionally identified based on the spatial correlation of probes with a significance threshold of 0.001 within a 1000 bp window, using comb-p. After Sidák correction, 27 DURs, 4 DMRs and 1 DHR were found to be significantly modified with the aggravation of tau pathology. Two genes (*DNAJB13*, *PLYRP1*) were shared between the unmodified and methylated datasets, whereas the remaining regions were unique. Detailed

presentation of these results can be found in Table 3.

WGCNA and GO

WGCNA was employed in order to characterize the association of DNA methylation changes with disease progression on a systems-level. As a general pipeline for each dataset, discrete modules of co(hydroxy)methylated positions were created and associated with Braak stage progression using the ME.

For the dataset with the UC, 384,911 probes were used as input and 39 modules were identified (Figure 3A). Thirteen modules were significantly correlated with Braak stage progression, but all failed to pass significance level after multiple testing correction (adjusted $p \leq 0.0013$). The most significant “salmon” module (4,035 probes, $r = -0.3$, nominal $P = 0.006$, $q = 0.23$) was correlated with probe significance, indicating an association between Braak stage progression and DNA methylation ($r = 0.07$, $p = 0.0032$; Figure 3B).

For the 5mC dataset, 389,204 probes were considered and 22 modules were identified (Figure 4A), from which only the “lightyellow” was significantly correlated with Braak stage progression, but failed to pass multiple testing adjustment of the p value (721 probes, $r = 0.2$, nominal $P = 0.04$, $q = 0.88$). Module membership within this module was correlated with probe significance ($r = 0.08$, $p = 0.034$; Figure 4B).

For the 5hmC dataset, 164,835 probes were considered and 6 modules were identified (Figure 5A). Four of them correlated with Braak stage progression, and the yellow module was the closest in surviving multiple testing correction (1,073 probes, $r = 0.05$, nominal $P = 0.01$, $q = 0.06$). Module membership within this module was correlated with probe significance ($r = -0.1$, $p = 0.002$; Figure 5B).

The core members of the aforementioned modules that displayed a high module membership (absolute $MM \geq 0.65$) were extracted for further examination of their biological significance. For the UC dataset, 690 probes in the salmon module were mapped to 495 unique genes. Highly enriched biological processes in this module included potassium transport-associated terms and postsynaptic cellular components. More specifically, pathways were related to cellular potassium ion transport processes (GO:0071804; nominal $P = 5.66E-05$, $p_{\text{bon}} = 0.19$ and postsynapse cellular components (GO:0098794; nominal $P = 0.00013$, $p_{\text{bon}} = 0.19$). For the 5mC dataset, 138 probes in the lightyellow module were mapped to 104 unique genes, involved in processes concerning regulation of synapse assembly (GO:0051965; nominal $P = 0.001$, $p_{\text{bon}} = 1$). Finally, 40 unique genes harbouring 58 probes in the yellow module of the 5hmC dataset were identified, and enriched processes within involved catenin regulation (GO:0035414; nominal $P = 0.0003$, $p_{\text{bon}} = 1$), membrane hyperpolarization nominal (GO:0060081; $P = 0.0005$, $p_{\text{bon}} = 1$) and cytokine production (GO:0002720; nominal $P = 0.0017$, $p_{\text{bon}} = 1$). In Table 4, the terms associated

with the aforementioned modules are presented.

Discussion

In the present study, we identified the AD-associated methylome and hydroxymethylome of the DRN, displaying unique epigenetic signatures among the different cytosine states. Interestingly, while studying the levels of UC, two previously unreported CpGs reached genome-wide significance. Furthermore, we discovered differentially epigenetically modified regions, clusters of significant CpGs, associated with the progression of AD, indicated by increasing Braak stages. Finally, several biological processes were found to be enriched in AD based on WGCNA and GO analysis.

The present study represents the first of its kind to characterize the methylomic and hydroxymethylomic profile of the DRN in AD. Where the great majority of previous DNA methylation studies in AD had made use of regular BS conversion and hence were unable to discriminate between DNA methylation from hydroxymethylation, the current approach specifically explored the methylome and hydroxymethylome, by means of parallel oxBS and BS treatment. Therefore, we were able to quantify the levels of three different cytosine states among the interrogated CpGs, and thus attempted to identify Braak stage-specific differences at these loci. Overall, it was observed that the three distinct cytosine states were outlined by largely different epigenetic profiles.

Focusing on unmodified (versus modified) cytosines, we reported two genes that showed significant differential levels of UC with disease progression, i.e. *IFFO2* and *PLCL2*. *IFFO2* (intermediate filament family orphan 2) is a gene associated with structural molecule activity and its expression is highly enriched in the brain, more specifically the cortex, hippocampus and cerebellum [29]. With respect to AD, it has been reported to be over-expressed in the frontal cortex of patients in comparison to neurologically normal controls [30], an effect attributed to regulation by microRNAs, i.e. miR-2110 and miR-214. Otherwise, little is known about this gene and its function in general. The fact that this *IFFO2* probe passes genome-wide significance levels urges for further research into its involvement in AD. Further, *PLCL2* (phospholipase C like 2), a phospholipase C-like protein that lacks phospholipase catalytic activity, is mainly associated with immune system functions [31]. Peripherally, it is expressed in hematopoietic cells and centrally in the hippocampus and to a smaller extent in the cortex, caudate and cerebellum [32]. Up to date, *PLCL2* has not been linked to any pathological neurological conditions. Interestingly, PLC-delta, a homologous enzyme to *PLCL2*, has been found to accumulate amidst neurofibrillary tangles in AD brains in advance of the aggregation of fibrillary deposits [33]. Among the other DUPs nearly reaching genome-wide significance after correcting for multiple testing, *HDAC4* was

ranked 5th. This histone deacetylase is a usual suspect in the list of genes/proteins associated with AD, as it is significantly overexpressed in the frontal cortex of AD patients [34]. Moreover HDAC inhibitors have been shown not to only rescue cognitive impairment but to exert pro-cognitive effects in preclinical studies [35]. The fact that its own epigenetic signature may be altered suggests an intricate multi-level dysregulatory epigenetic circuit in AD.

Reporting on differentially (hydroxy)methylated positions is useful for better understanding of the epigenetic machinery at the single base level, but interrogating differentially (hydroxy)methylated regions is more informative for identifying functional regions possibly involved in gene transcription regulation and deciphering the collective effect of DMPs in health and disease. Here, we report 27 DURs, 4 DMRs and one DHR associated with Braak stage progression. The top DUR spans 800 bp and the 22 DUPs included in this locus are within the coding sequence of *TNXB*. A more extended region of 1.3k bp, including the discovered DUR, has been previously reported in literature to be differentially unmodified [36] in the entorhinal cortex of AD patients from the same cohort used in this study. Such finding signifies the role of *TNXB* in the pathophysiology of AD. *TNXB* is a gene expressing tenascin-X (TN-X), a protein with anti-adhesive properties, initially linked to schizophrenia [37]. While its functional role in the brain has yet to be identified, a study has associated SNPs within the locus with progressive supranuclear palsy, a tau-related disorder [38]. Furthermore, a transcriptomic study associated four SNPs residing in the *TNXB* locus (rs3130285, rs3130286, rs185819, rs3130287) with increased risk of developing AD [39]. Among them, rs3130286, has been associated with AD in another, independent study [40]. Therefore, we strongly advocate that *TNXB* plays a critical role in the pathogenesis of AD, possibly affecting neuronal resilience to the tau-related insults, and further interrogation of the interplay between epigenetic modification and genetic variants shall shed more light in the exact function of this gene.

Other noteworthy DURs are found within the *CAST*, *RHBDL4*, *BMP6* and *BCAR3* genes. Calpastatins, the product of *CAST* gene, are known to protect against neuronal death induced by $A\beta$ [41, 42]. *RHBDL4* is responsible for efficiently cleaving APP inside the cell, inducing alternative processing bypassing amyloidogenesis and thereby reducing $A\beta$ levels [43]. *BMP6* is highly upregulated within the hippocampus of patients with severe AD impacting on neurogenesis, an observation speculated to be provoked by accumulation of $A\beta$ [44]. Finally, *BCAR3* has not only been associated general cognitive ability and memory [45], but, more importantly, a CpG (cg02342148) within the DUR was reported to be differentially methylated in AD based on the EWAS conducted by De Jager and

colleagues [16, 46].

Examining the DUR, DMR and DHR results collectively, we observe a couple of hits that are common among cytosine states. More specifically, two genetic loci were shared between the unmodified and methylated cytosine state, namely *DNAJB13* and *PGLYRP1*, underscoring an important role for active DNA demethylation. To this end, the same 4 probes within the *DNAJB13* locus have higher levels of unmodified and simultaneously decreased levels of methylated cytosines. *DNAJB13* is a member of the heat shock protein family 40 (Hsp40), proteins reported to impede the amyloidogenic cascade by preventing A β aggregation [47, 48]. The reported DUR within the *PGLYRP1* locus extends over 508 bp and 8 out of the 11 unmodified positions display decreased levels of methylation. Similarly, Lunnon and colleagues have described a slightly more extended region (576 bp) containing 10 probes within the same locus to be differentially unmodified with Braak stage progression in cortical areas of two independent cohorts [R]. *PGLYRP1* is an activator of TREM-1 [49], a protein capable, when overexpressed, to induce microglial-mediated A β clearance and restore AD-related cognitive impairment [50]. Whether there is a functional connection, direct or indirect, between the reported methylation patterns and TREM1 efficacy remains to be investigated.

WGCNA was used in order to identify clusters of highly correlated probes in each dataset that were then linked to disease progression. The probes from the most prominent modules created were then annotated for harbouring genes and GO analysis was performed in order to find biological processes disrupted in AD. Specifically, the highest ranked terms emerging from the UC dataset were related to potassium transport, a term previously described by De Jager to be altered in terms of methylation in AD [16]. Enriched terms emerging from the correlation analysis within the 5mC dataset involved synaptogenesis, a process known to be highly impaired in AD [51-54]. Finally, biological processes unveiled from the 5hmC dataset involved intranuclear catenin transport, membrane hyperpolarization as well as cytokine production. The transport of beta-catenin in the nucleus is the defining step for downstream gene expression in the Wnt/beta-catenin signalling pathway, a pathway largely known to be dysregulated in AD [55]. Cytokine production and especially IL-1 β is characterized as part of the neuropathological cascade of AD and has been linked to the pathological

APOE4 genotype [56].

Altogether, to our knowledge, this is the first study to use oxBS conversion in conjunction with the MethylationEPIC array in order to study the effect of Braak stage progression within the brainstem. Despite its relatively low samples size when compared to GWAS, this study undoubtedly is unique in its kind, presenting novel results, with some probed even passing genome wide significance levels. Notably, cut-off values applied after multiple testing correction might in fact be too conservative for EWAS, considering the high correlation among cis-CpG sites [57]. Hence, correcting for each one of them might lead to false negative results, a notion that is often overlooked.

An interesting outcome of this study is the demonstration of mostly unique “hits” that have not been reported in previous AD EWAS, conducted on cortical and hippocampal tissue. Such observation further advocates an explicit role of the brainstem in AD pathophysiology and a rather distinct epigenomic profile of the DRN in particular. To this end, the majority of the emerged genes have been biologically and functionally linked to AD and related pathology based on e.g. studies addressing genetic variation. According to our observations, we now speculate that epigenetic changes stemming from the tau-infested brainstem nuclei could initiate the deviant cascade of A β pathology. Further interrogation on the observed epigenetic changes in a spatiotemporal context will test this hypothesis and illuminate whether the observed epigenetic modifications are cause or consequence of these pathological hallmarks.

Moreover, the three distinct profiles of cytosine states reported in this study strengthen the notion that there are different functional roles attributed to each one of them and urge for further characterization of these modifications in health and disease. For a more comprehensive understanding of the biological function of each cytosine state of the genes emerging from this study as well as endorsement of the reported results, further studies with validation cohorts and/or targeted approached by means of pyrosequencing should be performed. Finally, the novel use of oxBS-based technology allowed for the segregation of methylation from hydroxymethylation and, therefore, it should be stressed that previously reported results, utilizing regular BS conversion technologies, should be interpreted with caution. Finally, it cannot be overlooked that the WGCNA analysis employed here and elsewhere [27] was conceptualized in order to create networks of co-expressed genes, and its suitability for assessing either multiple-probes-one-gene

data, like EPIC array datasets, or data of such length/volume (~340k probes) remains to be confirmed.

In conclusion, this study represents the first one to interrogate the methylome and hydroxymethylome of the brainstem in AD and to this end, we demonstrated a unique epigenomic profile of the DRN in association with Braak stage progression. Regardless the small sample size of the present study, two probes harboring genes previously associated with AD in the human brain survived multiple testing correction, prompting for further interrogation into the functional role of the methylomic changes within these loci. Furthermore, several of the identified DURLs and DMLs have been reported in different brain regions of the same cohort used here, indicating a systemic effect of these methylation signatures in advanced stages of AD. From a methodological perspective this study displays a bioinformatics pipeline for quality control and analysis of EWAS data is compatible with downstream data from the EPIC array and is applicable for all cytosine states. Altogether, the outcomes advocate the redirection of attention to brain structures closely related to the early presymptomatic stages of the disease, such as the brainstem as it outlines a unique profile of the disease previously unreported. Complementary studies focusing on distinct co-methylation networks and methylation quantitative trait loci across different brain regions, following the timeline of disease progression, are of pivotal importance in order to describe the biological phenomena of AD in a topographical yet chronological manner which will allow for deeper insight in the pathogenesis of the disease. Validating and deciphering the functional outcomes of the observations presented here would assist verifying the fundamental hypothesis that the brainstem is of pivotal importance for disease onset. Ultimately, a meticulous characterisation of the epigenome, genome and transcriptome of the brainstem will fill knowledge gaps concern the aetiopathogenesis of AD and in turn novel therapeutic as well as diagnostic targets will emerge, accelerating the developments towards treating and preventing this devastating disease.

Braak Stage	0	I	II	III	IV	V	VI
N	8	9	4	13	5	15	38
Sex (F/M)	6/2	4/5	2/2	7/6	4/1	8/7	21/17
Age at Death (Mean±SD)	73.25 ± 13.09	76.56 ± 10.78	85 ± 5.72	88.31 ± 5.09	89 ± 6.04	87.27 ± 8.28	84 ± 7.11
PMI (Mean ± SD)	29.5 ± 23.86	26.67 ± 16.22	39.75 ± 15.17	27.15 ± 19.59	43.8 ± 16.84	30.6 ± 22.22	36.76 ± 20.07

Table 1: Cohort demographics. Dorsal raphe nucleus tissue from individuals with different Braak stages (0-VI) was obtained from the London Brain Bank. The number of samples in each stage as well as the distribution of sex, age and postmortem interval

Probe ID	Estimate	p value	q value	Chromosome	Gene Name
Unmodified					
cg11237901	-0.0053	1.47E-09	0.001	1	IFFO2
cg22927063	0.0088	1.16E-07	0.043	3	PLCL2
cg20450401	0.0093	2.21E-06	0.486	13	LINC00371
cg23326220	0.0052	2.62E-06	0.486	19	KLK13
cg08565197	-0.0046	5.10E-06	0.750	2	HDAC4
cg06714901	0.0049	7.84E-06	0.750	12	CCDC63
cg16735404	0.0073	8.32E-06	0.750	1	-
cg08745506	-0.0063	1.06E-05	0.750	9	NEK6
cg05682333	0.0094	1.18E-05	0.750	2	ALPPL2
cg02905830	0.0049	1.35E-05	0.750	22	FAM19A5
Methylated					
cg19017674	0.014	1.79E-06	0.695	7	-
cg09989688	0.007	2.66E-06	0.695	11	C11orf94
cg16009115	0.011	3.29E-06	0.695	17	U6
cg03442014	0.003	4.65E-06	0.695	16	NDUFAB1
cg04044120	-0.021	7.26E-06	0.695	11	DNAJB13
cg22985466	-0.008	7.45E-06	0.695	12	PIP4K2C
cg05241973	0.008	7.50E-06	0.695	6	-
cg06420033	0.011	7.97E-06	0.695	8	ASH2L
cg21638110	0.004	9.13E-06	0.695	10	FAM53B
cg10840389	0.003	9.38E-06	0.695	10	LRRC20
Hydroxymethylated					
cg09177486	0.009	1.89E-06	0.303	1	NBPF20
cg25525535	-0.011	3.15E-06	0.303	20	CBFA2T2
cg02673938	-0.010	3.68E-06	0.303	19	KDM4B
cg06005688	0.010	3.85E-06	0.303	4	-
cg23544027	0.013	4.77E-06	0.303	4	PPID
cg10347136	-0.015	9.04E-06	0.418	8	NECAB1
cg21715339	-0.010	1.08E-05	0.418	1	TTC39A
cg16955752	0.014	1.11E-05	0.418	4	DCHS2
cg15368872	0.009	1.19E-05	0.418	12	KLRK1
cg08372227	0.008	1.43E-05	0.453	2	DNPEP

Table 2: The ten top-ranked Braak-associated differentially unmodified and modified (methylated and hydroxymethylated) positions. Displayed for each probe are the regression estimate for the Braak-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name)

Chromosome	Start	End	Nr Probes	p value	Šidák-p	Gene Name	Gene Feature	Change
Unmodified								
chr6	32063459	32064259	22	1.79E-13	1.75E-10	TNXB	cds	↑
chr11	73668348	73668762	4	2.43E-10	4.56E-07	DNAJB13	intron	↑
chr19	46526168	46526676	11	3.76E-10	5.76E-07	PGLYRP1	TSS+utr5+cds	↑
chr1	19110562	19111090	6	2.66E-09	3.92E-06	PAX7	intergenic	↑
chr1	3154081	3154701	5	7.42E-09	9.32E-06	PRDM16	intron	↓
chr1	3115309	3115645	4	7.54E-09	1.75E-05	PRDM16	intron	↓
chr14	24898818	24899167	11	2.29E-08	5.10E-05	KHNYN		↓
chr2	102051587	102051690	3	2.47E-08	0.00019	RFX8	intron	↓
chr15	90039582	90040047	12	1.33E-07	0.00022	RHCG		↓
chr6	90276624	90276834	7	6.62E-08	0.00025	ANKRD6	intron+utr5+cds	↓
chr3	35720994	35721285	7	1.06E-07	0.00028	ARPP21		↑
chr5	95997186	95997356	4	6.46E-08	0.00030	CAST	intergenic	↑
chr18	72837386	72837783	7	2.21E-07	0.00043	ZNF407	intergenic	↑
chr15	28351607	28351907	3	1.87E-07	0.00048	HERC2	intergenic	↑
chr12	6933322	6933549	5	3.70E-07	0.00127	GPR162		↑
chr7	2519789	2519875	3	2.34E-07	0.00212	GRIFIN	intergenic	↑
chr2	98340425	98340656	3	8.19E-07	0.00276	ZAP70	intron+utr5+cds	↑
chr12	48399140	48399296	4	5.78E-07	0.00288	COL2A1	intergenic	↓
chr19	3482641	3482934	4	1.12E-06	0.00298	SMIM24	intergenic	↑
chr1	94050704	94050904	4	9.05E-07	0.00352	BCAR3		↑
chr7	38350921	38351227	5	2.05E-06	0.00519	TRG-AS1	intergenic	↑
chr7	1152070	1152402	4	2.43E-06	0.00568	C7orf50		↑
chr16	67204216	67204448	8	1.81E-06	0.00606	NOL3	TSS+exon+utr5	↓
chr10	135061171	135061487	9	2.56E-06	0.00629	MIR202HG	nc_intron+nc_exon	↑
chr1	39395165	39395390	4	1.99E-06	0.00685	RHBDL2		↓
chr20	55799852	55800008	4	1.52E-06	0.00753	BMP7	intron	↓
chr13	100217924	100217996	3	1.01E-06	0.01085	TM9SF2	intergenic	↑
Methylated								
chr11	73668348	73668762	4	1.15E-13	2.16E-10	DNAJB13	intron	↓
chr16	2867434	2868002	6	6.65E-11	9.11E-08	PRSS21		↓
chr6	32118204	32118458	13	6.32E-08	0.00019	PRRT1	cds	↓
chr19	46526321	46526652	8	8.74E-06	0.02035	PGLYRP1	TSS+exon+utr5	↓
Hydroxymethylated								
chr1	151810586	151810905	6	3.03E-09	3.13E-06	C2CD4D	cds	↑

Table 3: Identification of probes in differentially unmodified and modified (methylated and hydroxymethylated) regions associated with Braak stage score. Displayed for each probe are the chromosomal position (genome build 37), the number of probes in the region (Nr Probes), the p-value and multiple testing-corrected p (Šidák-p), UCSC gene name, gene feature, and directionality of modification changes with the progression of Braak stage (up or down). TSS, transcription start site; utr5, 5' untranslated region; cds, coding sequence.

Figure 1: Unmodified cytosine (UC) levels with the progression of Braak stage score. Regressed UC values of the two top-ranked probes, cg11237901 and cg22927063, residing within the IFFO2 and PLCL2 genetic loci, respectively.

Figure 2: Manhattan plots of the unmodified, methylated and hydroxymethylated cytosine regression analyses. The blue line indicates the nominal significance threshold of 0.05 and the red one illustrates the genome-wide significance threshold.

Figure 3: Probe modules of co-unmodified cytosines with the progression of Braak stage. (A) Heatmap representing the correlation between module eigenvalues (ME) and Braak stage, age as well as sex. Each row represents a module, as indicated on the y axis and each column a trait, namely Braak stage score, age and sex. As shown in the color scale bar, strong positive correlation is indicated by dark red, strong negative correlation is indicated by dark blue, and white indicates no correlation. Each cell contains the correlation coefficient and the p value given in the parentheses. The salmon module is the most significantly associated with the progression of Braak stage ($p = 0.006$). (B) The module membership is correlated with probe significance for Braak stage score ($r = 0.071$, $p = 0.0032$).

A

B

Figure 4: Probe modules of co-methylated cytosines with the progression of Braak stage. Heatmap representing the correlation between module eigenvalues (ME) and Braak stage, age as well as sex. Each row represents a module, as indicated on the y axis and each column a trait, namely Braak stage score, age and sex. As shown in the color scale bar, strong positive correlation is indicated by dark red, strong negative correlation is indicated by dark blue, and white indicates no correlation. Each cell contains the correlation coefficient and the p value given in the parentheses. The Lightyellow module is associated with the progression of Braak stage ($p = 0.04$). Furthermore, the module membership is correlated with probe significance for Braak stage score ($r = 0.079$, $p = 0.034$).

Figure 5: Probe modules of co-hydroxymethylated cytosines with the progression of Braak stage. Heatmap representing the correlation between module eigenvalues (ME) and Braak stage, age as well as sex. Each row represents a module, as indicated on the y axis and each column a trait, namely Braak stage score, age and sex. As shown in the color scale bar, strong positive correlation is indicated by dark red, strong negative correlation is indicated by dark blue, and white indicates no correlation. Each cell contains the correlation coefficient and the p value given in the parentheses. The Yellow module is associated with the progression of Braak stage ($p = 0.01$). Furthermore, the module membership is correlated with probe significance for Braak stage score ($r = 0.096$, $p = 0.0016$).

Rank	ID	Term	Type	Genes in Term	Genes in input list and term	p value	q value	Genes names in input list and term
Unmodified								
1	GO:0071804	cellular potassium ion transport	BP	181	18	5.66E-05	0.19	HCN4, CNGB1, KCNU1, KCNG3, DPP6, KCNH4, KCNIP3, KCNA2, KCNN1, KCNQ3, ATP1A3, SLC9A3, CACNA1D, KCNH6, KCNAB2, KCNK17, KCNH7, NOS1AP
2	GO:0071805	potassium ion transport	BP	210	19	0.000121425	0.19	HCN4, CNGB1, ADORA1, KCNU1, KCNG3, DPP6, KCNH4, KCNIP3, KCNA2, KCNN1, KCNQ3, ATP1A3, SLC9A3, CACNA1D, KCNH6, KCNAB2, KCNK17, KCNH7, NOS1AP
3	GO:0006813	potassium channel activity	MF	119	13	0.000124374	0.19	HCN4, CNGB1, KCNU1, KCNG3, KCNH4, KCNIP3, KCNA2, KCNN1, KCNQ3, KCNH6, KCNAB2, KCNK17, KCNH7
4	GO:0005257	postsynapse	CC	339	29	0.000126772	0.19	CHRM3, ADORA1, SLC29A1, EPHA4, SHANK2, IGSF9B, NCS1, FYN, D45234E, GRID1, GRIK3, GRIK4, LYN, ATP1A3, NTSR1, ASAP1, PALMD, BAI1, MYL7, CACNG8, CPEB1, LRPI8, AXIN1, AXIN2, DTNBP1, KCNAB2, ITGA8, HTR3B, RICH2
5	GO:0069794	potassium ion transmembrane transporter activity	MF	151	15	0.000144293	0.19	HCN4, CNGB1, KCNU1, KCNG3, KCNH4, KCNIP3, KCNA2, KCNN1, KCNQ3, ATP1A3, SLC9A3, KCNH6, KCNAB2, KCNK17, KCNH7
6	GO:0069572	postsynaptic density	CC	143	16	0.000513731	0.46	ADORA1, EPHA4, SHANK2, IGSF9B, NCS1, FYN, LYN, BAI1, CACNG8, CPEB1, LRPI8, AXIN1, AXIN2, DTNBP1, KCNAB2, ITGA8
7	GO:0042391	voltage-gated potassium channel activity	MF	88	10	0.00056141	0.46	HCN4, CNGB1, KCNU1, KCNG3, KCNH4, KCNA2, KCNQ3, KCNH6, KCNAB2, KCNH7
8	GO:0022843	regulation of membrane potential	BP	342	24	0.000576207	0.46	HCN4, CFTR, C9orf7, SLC26A9, CNGB1, ADORA1, SLC29A1, IGSF9B, KCNH4, ABL1, GRIK3, KCNA2, KCNQ3, ATP1A3, NTSR1, PARK2, PID1, BRUNOL4, YWHAH, CACNA1D, KCNH6, KCNK17, KCNH7, NOS1AP
9	GO:0022836	cation channel complex	CC	170	16	0.000761951	0.56	CNGB1, KCNU1, KCNG3, DPP6, KCNH4, KCNIP3, KCNA2, KCNN1, KCNQ3, PDE4D, CATSPERG, CACNG8, CACNA1D, KCNAB2, HTR3B, NOS1AP
10	GO:0060076	voltage-gated cation channel activity	MF	131	13	0.000990421	0.61	HCN4, CNGB1, KCNU1, KCNG3, NCS1, KCNH4, KCNA2, KCNQ3, CACNG8, CACNA1D, KCNH6, KCNAB2, KCNH7
Methylated								
1	GO:0043218	compact myelin	CC	15	2	0.004489815	1	MAG, MBP
2	GO:0044088	positive regulation of CC biogenesis	BP	426	8	0.00495236	1	TPPP, CUX2, HTT, BAI1, THBS2 NR1H2, NRXN3, FAM38A
3	GO:0030424	axon	CC	401	8	0.006521451	1	STX6, HEPACAM, HTT, KCNB1, MAG, MBP, PTPRN1, PTPRN2
4	GO:0030261	chromosome condensation	BP	34	2	0.013763243	1	POLS, CDK1
5	GO:0019932	second-messenger-mediated signaling	BP	234	5	0.015554854	1	DDAH2, HTT, NFATC1, MCTP2, NOS1AP
6	GO:0032657	axon part	CC	208	5	0.015606925	1	STX6, MAG, MBP, PTPRN1, PTPRN2
7	GO:0006636	response to toxic substance	BP	201	4	0.016342663	1	GPX6, MBP, TXNRD1, CDK1
8	GO:0007416	synapse assembly	BP	135	4	0.01948497	1	CUX2, BAI1, THBS2, NRXN3
9	GO:0007030	Golgi organization	BP	99	3	0.024648676	1	STX6, HTT, CDK1
10	GO:0046824	positive regulation of nucleocytoplasmic transport	BP	112	3	0.024872243	1	KHDRBS1, SEMA5A, CDK1
Hydroxymethylated								
1	GO:0035414	negative regulation of catenin import into nucleus	BP	10	2	0.000321502	1	DAB2IP, WWTR1
2	GO:0060081	membrane hyperpolarization	BP	12	2	0.000530829	1	KCNQ3, PRKCZ
3	GO:0002720	positive regulation of cytokine production involved in immune response	BP	31	2	0.00178591	1	PRKCZ, SEMA7A
4	GO:0007225	integrin-mediated signaling pathway	BP	88	3	0.003166875	1	DOCK1, ITGA6, SEMA7A
5	GO:0071886	14-3-3 protein binding	MF	23	2	0.003750993	1	DAB2IP, PRKCZ
6	GO:0010811	positive regulation of cell-substrate adhesion	BP	102	3	0.004092219	1	DOCK1, ITGA6, PRKCZ
7	GO:0010596	negative regulation of endothelial cell migration	BP	39	2	0.004538363	1	DAB2IP, PTPRM
8	GO:0031463	Cu3-RING ubiquitin ligase complex	CC	65	2	0.008477305	1	KLHL30, KCTD10
9	GO:0010486	proteasomal protein catabolic process	BP	402	4	0.01514252	1	LOC400927-CSNK1E, DAB2IP, WWTR1, KCTD10
10	GO:0010532	regulation of epithelial cell migration	BP	166	3	0.015411362	1	DAB2IP, DOCK1, PTPRM

Table 4: Gene Ontology (GO) enrichment analysis results for the probes included in the most significant modules for each cytosine state (salmon for unmodified; lightyellow for methylated; yellow for hydroxymethylated). Each GO term is presented along with the ID, name, type, the number of genes in the pathway and the represented ones in the input list, the p-value, the FDR-corrected p value (q value) as well as the names of the genes. Displayed are the 10 highest ranked terms based on p-value for each cytosine state. BP; Biological Process, MF; Molecular Function, CC; Cellular Component

Normalization metrics

	All processed and metrics together			All processed together then metrics on BS only			All processed together then metrics on oxBS only			Processed and metrics on BS only			Processed and metrics on oxBS only		
	Type I	Type II	Combined	Type I	Type II	Combined	Type I	Type II	Combined	Type I	Type II	Combined	Type I	Type II	Combined
raw	9	8.667	9.333	9	12.333	9	8.667	11.667	11	9.333	12.333	9.333	9	12	10
dasen	4.333	2.667	3.667	3.667	3.667	3	2.667	2	1.333	2.667	3.333	3	2.333	4	3
betaqn	12	10.333	11	11	9	10	14	11.667	12	13.333	11	12	13	10.333	12
naten	5	6.333	6	7.333	5	6	6.667	3	6.667	7.333	5	6	8	5	8.333
nanet	6.667	10.333	9	9.667	9	8.333	8.667	6.333	8.667	8.667	8.667	8	8.667	6.667	7.667
nanes	8.333	9.667	8.333	6	6.667	6.333	8.333	8	8.333	6.667	6.333	6	8	7.667	7
danes	5.333	8.667	7	4	5.667	5.667	3.667	7	5	3.667	5.333	4.667	3.333	6.667	3.667
danet	7.667	9	8.333	11.333	7.333	10	7.667	8	7	10.667	7	9.333	8.333	6.333	6.667
danen	6.333	8.333	8	4.333	11.667	8	5.333	11.333	8.667	3.667	11.333	8	6.667	11.333	8
daten1	6.667	3.667	5	9.333	4	7.667	7.333	6.667	6	9	4.333	7	5.667	5.333	7.333
daten2	6.333	4	4.333	10.333	3.667	8	8.333	7.333	6.333	10	3.667	7.333	7.333	5.667	7.667
nasen	9.333	3.667	5.667	5	4.667	3	7.333	3	4.333	5.667	4.333	4	7	5	4.667
fuks	10	10.333	10.333	9.333	13.667	13.667	9.667	13.667	13.667	9.667	13.667	13.667	10	13.667	13.667
tost	8	9.333	9	4.667	8.667	6.333	6.667	5.333	6	4.667	8.667	6.667	7.667	5.333	5.333
best	dasen	dasen	dasen	dasen	dasen/daten2	dasen/nasen	dasen	dasen	dasen	dasen	dasen	dasen	dasen	dasen	dasen

Supplementary Table 1: Overall rank scores of each watermelon preprocessing normalization method. The test metrics were used to quantitatively determine the best performing preprocessing method in comparison to raw data from raw bisulfite

Supplementary Figure 1: Density plots of normalized beta values for unmodified (A), methylated (B) and hydroxymethylated (C) cytosines. Plots include all probes used for the analysis. The unmodified beta values are computed by subtracting the normalized beta values obtained from bisulfite converted DNA (BS; containing signals from both 5-mC and 5-hmC), from 1. The methylation beta values directly correspond to the normalized beta values from oxidative bisulfite converted DNA (oxBS), and finally the hydroxymethylation beta values are determined by subtracting the normalized oxBS beta values from the BS beta values and considering solely the probes that display significantly higher average BS when compared to oxBS levels, as assessed using a paired t-test.

Supplementary Figure 2: Correlation matrix of the identified surrogate variables (sv) and variables known to affect methylation status. Color and size-coded circles on the upper right diagonal represent the correlation coefficients shown in the lower left diagonal of the table. Strong positive correlation is indicated by blue color and on the contrary, strong negative correlation by red. BS, beta values from bisulfite converted DNA; N_pos, NeuN positive cells representing neurons; N_neg, NeuN negative cells representing glial cells.

Supplementary Figure 3: QQ plots of the unmodified (A), methylated (B) and hydroxymethylated (C) cytosine regression analyses. The fit of each model was evaluated by the genomic inflation factor lambda (λ), which is depicted on the right side of each plot

References

1. Organization, W.H. Dementia: Fact sheet. 2017; Available from: <http://www.who.int/mediacentre/factsheets/fs362/en/>.
2. Murphy, M.P. and H. LeVine, 3rd, Alzheimer's disease and the amyloid-beta peptide. *J Alzheimers Dis*, 2010. 19(1): p. 311-23.
3. Iatrou, A., et al., Epigenetic dysregulation of brainstem nuclei in the pathogenesis of Alzheimer's disease: looking in the correct place at the right time? *Cellular and Molecular Life Sciences*, 2017. 74(3): p. 509-523.
4. Seeley, W.W., et al., Neurodegenerative diseases target large-scale human brain networks. *Neuron*, 2009. 62(1): p. 42-52.
5. Braak, H. and E. Braak, Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol*, 1991. 82(4): p. 239-59.
6. Korczyn, A.D., Why have we failed to cure Alzheimer's disease? *J Alzheimers Dis*, 2012. 29(2): p. 275-82.
7. Theofilas, P., et al., Turning on the Light Within: Subcortical Nuclei of the Isodentritic Core and their Role in Alzheimer's Disease Pathogenesis. *J Alzheimers Dis*, 2015. 46(1): p. 17-34.
8. Theofilas, P., et al., Locus coeruleus volume and cell population changes during Alzheimer's disease progression: A stereological study in human postmortem brains with potential implication for early-stage biomarker discovery. *Alzheimer's & Dementia*, 2017. 13(3): p. 236-246.
9. Theofilas, P., et al., Probing the correlation of neuronal loss, neurofibrillary tangles, and cell death markers across the Alzheimer's disease Braak stages: a quantitative study in humans. *Neurobiology of Aging*, 2018. 61: p. 1-12.
10. Grinberg, L.T., et al., The dorsal raphe nucleus shows phospho-tau neurofibrillary changes before the transentorhinal region in Alzheimer's disease. A precocious onset? *Neuropathol Appl Neurobiol*, 2009. 35(4): p. 406-16.
11. Gatz, M., et al., Role of genes and environments for explaining Alzheimer disease. *Archives of general psychiatry*, 2006. 63(2): p. 168-174.
12. Ridge, P.G., et al., Alzheimer's Disease: Analyzing the Missing Heritability. *PLOS ONE*, 2013. 8(11): p. e79771.
13. Lardenoije, R., et al., The epigenetics of aging and neurodegeneration. *Prog Neurobiol*, 2015. 131: p. 21-64.

14. Ziller, M.J., et al., Charting a dynamic DNA methylation landscape of the human genome. *Nature*, 2013. 500(7463): p. 477-481.
15. Lunnon, K., et al., Variation in 5-hydroxymethylcytosine across human cortex and cerebellum. *Genome biology*, 2016. 17(1): p. 27.
16. De Jager, P.L., et al., Alzheimer's disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDF2 and other loci. *Nature neuroscience*, 2014. 17(9): p. 1156.
17. Lunnon, K., et al., Methylomic profiling implicates cortical deregulation of ANK1 in Alzheimer's disease. *Nature neuroscience*, 2014. 17(9): p. 1164.
18. Davis, S., et al., methylumi: Handle Illumina methylation data. R package version, 2014. 2(0).
19. Schalkwyk, L., et al., watermelon: Illumina 450 methylation array normalization and metrics. R package version, 2013. 1(2).
20. Blair, J.D. and E.M. Price, Illuminating potential technical artifacts of DNA-methylation array probes. *The American Journal of Human Genetics*, 2012. 91(4): p. 760-762.
21. Chen, Y.-a., et al., Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics*, 2013. 8(2): p. 203-209.
22. Wheldon, L.M., et al., Transient accumulation of 5-carboxylcytosine indicates involvement of active demethylation in lineage specification of neural stem cells. *Cell reports*, 2014. 7(5): p. 1353-1361.
23. Skvortsova, K., et al., Comprehensive evaluation of genome-wide 5-hydroxymethylcytosine profiling approaches in human DNA. *Epigenetics & Chromatin*, 2017. 10: p. 16.
24. Leek, J.T. and J.D. Storey, Capturing heterogeneity in gene expression studies by surrogate variable analysis. *PLoS genetics*, 2007. 3(9): p. e161.
25. Pedersen, B.S., et al., Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. *Bioinformatics*, 2012. 28(22): p. 2986-2988.
26. Langfelder, P. and S. Horvath, WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics*, 2008. 9(1): p. 559.

27. Spiers, H., et al., Methylomic trajectories across human fetal brain development. *Genome research*, 2015. 25(3): p. 338-352.
28. Phipson, B., J. Maksimovic, and A. Oshlack, missMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform. *Bioinformatics*, 2015. 32(2): p. 286-288.
29. Uhlén, M., et al., Tissue-based map of the human proteome. *Science*, 2015. 347(6220): p. 1260419.
30. Bennett, J.P. and P. Keeney, Micro RNA's (miRNA's) may help explain expression of multiple genes in Alzheimer's Frontal Cortex. *J Syst Integr Neurosci*, 2017. 3(5): p. 1-9.
31. Takenaka, K., et al., Role of phospholipase C-L2, a novel phospholipase C-like protein that lacks lipase activity, in B-cell receptor signaling. *Molecular and cellular biology*, 2003. 23(20): p. 7329-7338.
32. Hirokawa, M., et al., A genome-wide association study identifies PLCL2 and AP3D1-DOT1L-SF3A2 as new susceptibility loci for myocardial infarction in Japanese. *European Journal of Human Genetics*, 2015. 23(3): p. 374-380.
33. Shimohama, S., et al., Aberrant accumulation of phospholipase C-delta in Alzheimer brains. *The American journal of pathology*, 1991. 139(4): p. 737.
34. Shen, X., et al., Neurons in Vulnerable Regions of the Alzheimer's Disease Brain Display Reduced ATM Signaling. *eNeuro*, 2016. 3(1): p. ENEURO.0124-15.2016.
35. Gräff, J. and L.-H. Tsai, The potential of HDAC inhibitors as cognitive enhancers. *Annual review of pharmacology and toxicology*, 2013. 53: p. 311-330.
36. Lunnon, K., et al., Cross-tissue methylomic profiling strongly implicates a role for cortex-specific deregulation of ANK1 in Alzheimer's disease neuropathology. *Nature neuroscience*, 2014. 17(9): p. 1164-1170.
37. Wei, J. and G. Hemmings, TNXB locus may be a candidate gene predisposing to schizophrenia. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2004. 125(1): p. 43-49.
38. T., C., Tau Network Genes in a Genome Wide Association Study of Progressive Supranuclear Palsy. 2017: *Mov Disord*.

39. Chapuis, J., et al., Transcriptomic and genetic studies identify IL-33 as a candidate gene for Alzheimer's disease. *Molecular psychiatry*, 2009. 14(11): p. 1004.
40. Sherva, R., et al., Identification of Novel Candidate Genes for Alzheimer Disease by Autozygosity Mapping Using Genome Wide SNP Data From an Israeli-Arab Community. *Journal of Alzheimer's disease : JAD*, 2011. 23(2): p. 10.3233/JAD-2010-100714.
41. Nakayama, J., et al., Mutation analysis of the calpastatin gene (CAST) in patients with Alzheimer's disease. *Neuroscience Letters*, 2002. 320(1): p. 77-80.
42. Nixon, R.A., et al., Calcium-activated neutral proteinase (calpain) system in aging and Alzheimer's disease. *Ann N Y Acad Sci*, 1994. 747: p. 77-91.
43. Paschkowski, S., et al., Alternative Processing of the Amyloid Precursor Protein Family by Rhomboid Protease RHBDL4. *The Journal of Biological Chemistry*, 2016. 291(42): p. 21903-21912.
44. Crews, L., et al., Increased BMP6 levels in the brains of Alzheimer's disease patients and APP transgenic mice are accompanied by impaired neurogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 2010. 30(37): p. 12252-12262.
45. Hill, W.D., et al., Human cognitive ability is influenced by genetic variation in components of postsynaptic signalling complexes assembled by NMDA receptors and MAGUK proteins. *Translational psychiatry*, 2014. 4(1): p. e341.
46. Smith, A.R., et al., Elucidating novel dysfunctional pathways in Alzheimer's disease by integrating loci identified in genetic and epigenetic studies. *Neuroepigenetics*, 2016. 6(Supplement C): p. 32-50.
47. Broer, L., et al., Association of HSP70 and its co-chaperones with Alzheimer's Disease. *Journal of Alzheimer's disease : JAD*, 2011. 25(1): p. 93-102.
48. Carnini, A., et al., Cell Line Specific Modulation of Extracellular A β 42 by Hsp40. *PLOS ONE*, 2012. 7(5): p. e37755.
49. Read, C.B., et al., Cutting Edge: Identification of Neutrophil PGLYRP1 as a Ligand for TREM-1. *The Journal of Immunology*, 2015. 194(4): p. 1417.
50. Saadipour, K., TREM1: A Potential Therapeutic Target For Alzheimer's Disease. *Neurotox Res*, 2017. 32(1): p. 14-16.

51. Duyckaerts, C., B. Delatour, and M.C. Potier, Classification and basic pathology of Alzheimer disease. *Acta Neuropathol*, 2009. 118(1): p. 5-36.
52. Koffie, R.M., et al., Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. *Proc Natl Acad Sci U S A*, 2009. 106.
53. Lacor, P.N., et al., Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci*, 2007. 27.
54. Yoshiyama, Y., et al., Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron*, 2007. 53.
55. Vallée, A. and Y. Lecarpentier, Alzheimer disease: crosstalk between the canonical Wnt/beta-catenin pathway and PPARs alpha and gamma. *Frontiers in neuroscience*, 2016. 10.
56. Olgati, P., et al., APOE epsilon-4 allele and cytokine production in Alzheimer's disease. *Int J Geriatr Psychiatry*, 2010. 25(4): p. 338-44.
57. Shoemaker, R., et al., Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome. *Genome Research*, 2010. 20(7): p. 883-889.

4 Epigenetic variation of the miR-137 gene in Alzheimer's disease

Abstract	p.110
Keywords	p.110
Introduction	p.111
Material and Methods	p.112
Patient cohorts	p.112
BSHRI Cohort	p.112
AgeCoDe Cohort	p.112
Methylation data acquisition and analysis	p.113
BSHRI Cohort	p.113
AgeCoDe Cohort	p.113
Gene expression data acquisition and analysis	p.114
Results	p.114
DNA Methylation	p.114
MTG miR-137 expression in AD	p.115
Discussion	p.115
References	p.128

Artemis Iatrou¹, Roy Lardenoije¹, J. A. Y. Roubroeks^{1,2}, Ehsan Pishva^{1,2}, Diego Mastroeni^{1,3}, Paul D. Coleman³, Alfredo Ramirez^{1,5}, Bart P. F. Rutten¹, Gunter Kenis¹, Daniel L. A. van den Hove^{1,6}

1 School for Mental Health and Neuroscience (MHeNs), Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, the Netherlands

2 University of Exeter Medical School, University of Exeter, Exeter, UK

3 ASU-Banner Neurodegenerative Dis. Res. Ctr., Arizona State University, Tempe, AZ, USA

4 Institute of Human Genetics, University of Bonn, 53127 Bonn, Germany.

5 Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Wuerzburg, Germany

Abstract

MicroRNAs (miRNAs) are small noncoding RNAs primarily involved in the posttranscriptional control of gene expression. Recently, their critical role in neurodevelopment and aging has gained momentum, associating them with Alzheimer's disease (AD). MiR-137 has a functional role in presynaptic vesicle release as well as synaptic strength within the adult hippocampus and its expression is regulated by genetic factors as well as epigenetic modifications. Genetic variants of miR-137 have been linked to severe cognitive impairment in schizophrenia. In AD, decreased expression of miR-137 has been associated with pronounced amyloid beta ($A\beta$) production, one of the main hallmarks of the disease. Here, we investigated the role of miR-137 in AD and more specifically the locus-specific methylomic changes in AD patients. Making use of Illumina's HumanHT-12 v4 BeadChip and Infinium 450K Beadarray, we profiled the expression and methylation patterns of MIR137, respectively, in brains of AD patients and age-matched controls. Additionally, we interrogated blood methylation changes of MIR137 over time in a cohort of healthy aged individuals, some of which that converted to AD. Our findings show subtle methylation changes of MIR137 in the blood of AD patients as well as a significant association between MTG methylation and AD hallmark aggravation. Furthermore, we show that miR-137 expression is highly affected by the post mortem interval of the processed tissue, suggesting that miR-137 mRNA has a rather fast decay rate. Altogether, our study is the first one to investigate the methylomic profile of MIR137 in the brain and blood of AD patients, fundamentally indicating that methylation of the specific locus is a stable marker, regardless the post mortem interval variation. Specifically the reported MIR137 methylation patterns are correlated with AD pathology centrally and there is a tendency towards decreased global methylation of MIR137 with AD diagnosis peripherally.

Keywords: Alzheimer's disease; microRNA; miR-137; Epigenetics; DNA methylation

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs, 18-22-nucleotide long, functioning as endogenous master regulators of gene expression [1] and thus are fundamental components of the epigenetic machinery. MiRNA expression is in turn regulated by epigenetic mechanisms, including DNA methylation. MiRNAs are implicated in a wide range of biological functions, including neurogenesis, neuronal development and differentiation, as well as synaptic plasticity [2, 3]. Such processes are highly altered in pathological conditions, hinting at a generalized involvement of miRNAs in various neuropsychiatric and neurodegenerative disorders, such as Alzheimer's disease (AD) [4, 5]. MiR-137, the expression of which is enriched in the brain and especially the dentate gyrus (DG) of the hippocampus, is located at 1p21.3 within the noncoding RNA gene AK311400, embedded in a CpG island (Figure 1) [5-7]. Its expression has been reported to be regulated by an epigenetic-based system consisting of methyl-CpG binding protein 2 (MeCP2), the transcription factor Sox2, and the histone methyltransferase enhancer of zeste homolog 2 (Ezh2) [8, 9].

To date, the *MIR137* host gene (*MIR137HG*) locus has attracted a lot of attention as genome-wide association studies (GWAS) have identified genetic variants in miR-137 that are associated with cognitive impairment in schizophrenia [3]. Additionally, lower episodic as well as working memory performance and general cognitive capacity have been associated with the *MIR137* locus [10]. To this end, decreased levels of miR-137 induced by chromosomal microdeletion of 1p21.3 have been reported to be associated with intellectual disability [11]. Concerning AD, Geekiyanage and colleagues showed decreased central and peripheral miR-137 expression in sporadic AD patients, that they later associated with increased levels of ceramide, a lipid known to increase beta amyloid ($A\beta$) production [12, 13].

In the present study, we attempted to provide insight in the role of miR-137 in AD and, more specifically, interrogate how epigenetic variation in *MIR137* may contribute to the disease. To this end, we investigated methylation and expression patterns of the *MIR137* locus in the mediotemporal gyrus (MTG), a brain region affected by AD pathology. Furthermore, we evaluated longitudinal DNA methylation changes of *MIR137* in blood cells from a large aging cohort of elderly (>75 years) who had no signs of dementia at baseline and whose progression towards dementia was monitored over time [14]. Using this design, converters that developed dementia at follow-up examinations, and non-converting controls could be compared, both longitudinally even including a stage before the presence of clinical dementia, to gain insight into the potential use of blood *MIR137* methylation as a biomarker for AD.

Material and Methods

Patient cohorts

Brain and blood samples were provided by the Banner Sun Health Research Institute (BSHRI) in Sun City, Arizona, U.S.A., and the German Study on Ageing, Cognition and Dementia in Primary Care Patients (AgeCoDe), respectively, as described below. The medical ethics committee at each centre approved the relevant studies. All patients provided informed consent. Detailed demographic specification of the samples used can be found in Table 1.

BSHRI Cohort

Post-mortem middle temporal gyrus (MTG) brain tissue from AD and neurologically normal controls was obtained from the Brain and Body Donation Program by the BSHRI. A total of 80 post-mortem DNA samples (39 males, 41 females) were analysed. Participants were followed over the course of several years before death. AD diagnostic criteria followed guidelines for the National Institute on Aging (NIA)-Reagan Institute criteria (The National Institute on Aging & Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997). Post-mortem diagnosis, plaque and tangle load as well as Braak stage [15] had been collected by certified neurologists and neuropsychologists. Exclusion criteria included comorbidity with any other type of dementia, cerebrovascular disorders, brain trauma, or any disorder related to cognitive impairment (such as schizophrenia or Parkinson's disease).

AgeCoDe Cohort

Demographic information as well as whole blood DNA methylation data from 97 participants (27 males and 70 females) were provided by AgeCoDe [16] cohort. All individuals aged 75 years and older were non-demented at baseline. Of these, 55 converted and were thus diagnosed with AD at follow-up, after ~4.5 years and in case of 13 individuals up to 11 years after baseline [17].

Methylation data acquisition and analysis

BSHRI Cohort

Bisulfite (BS) and oxidative bisulfite (oxBS)-treated genomic DNA (gDNA) extracted from the MTG with standard phenol-chloroform protocol, was amplified and hybridized on the HumanMethylation450k BeadChip (Illumina, Inc., San Diego, CA, U.S.A.), and the Illumina iScan was used for imaging of Infinium BeadArray. Sample preparation, hybridization, and washing steps for the Illumina Infinium II Methylation Assay of the BeadChip arrays were performed according to the manufacturer's protocol. The raw beta values were extracted and normalized with *dasen* from the *watermelon* package [18]. The two treatments (BS, oxBS) applied allow the discrimination of three cytosine states: unmodified ($1-\beta$ BS), methylated (β oxBS) and hydroxymethylated (β BS- β oxBS). From each of the three resulting dataset 5 probes annotating the *MIR137* locus (cg04293733, cg05423529, cg10489614, cg14783814, cg22333214; Figure 1) were extracted. Linear regression was used to evaluate the levels of MTG *MIR-137* locus cytosine modifications between AD patients and controls and p values were adjusted with Bonferroni correction test. A correlation test was performed to evaluate the association of methylation of each probe and expression of the *miR-137*. Additionally, correlation tests were ran between each tested probe and PMI, plaque load as well as tangle load separately. The regression models designed were corrected for sex and age as well as 5 surrogate variables (SVs) that were detected from surrogate variable analysis (SVA) [19].

AgeCoDe Cohort

BS-treated gDNA was extracted from blood samples with standard phenol-chloroform protocol. As previously, gDNA was amplified and hybridized on the HumanMethylation450k BeadChip according to the manufacturer's protocol. Upon normalization, the 5 probes harbouring the *MIR137* locus were extracted. Once the data frame was split by time point, to baseline and T1, two regression models assessing the methylation levels of *miR-137* between AD converters and controls at baseline and T1 (4.5 years later) corrected for age and sex and 5 SVs were created. Accordingly, mixed model regression analysis was performed with the *nlme* package [20] in order to evaluate peripheral *MIR-137* methylation changes with disease diagnosis overtime. Multiple testing correction was performed with the Bonferroni correction test.

Gene expression data acquisition and analysis

Raw MTG expression data for the miR-137 probe was provided by the BSHRI using Illumina's HumanHT-12 v4 BeadChip. Total RNA extracted from frozen MTG was isolated with RNEasy Mini Kit (Qiagen) starting with at least 60 mg of tissue. The raw data was exported from Illumina's GenomeStudio (version 2011.1) with the Expression Module (v1.9.0) for further analysis in R. Of the 82 subjects passing the exclusion criteria, 1 case was not included on the expression array, and 3 additional cases were excluded after quality control of the data, due to extreme outlying values or failed reads, leaving 78 subjects for further analyses. Data was further normalized using the lumi package [21]. A linear regression model corrected for sex, age and post mortem interval (PMI) was applied in order to evaluate the miR-137 expression with disease diagnosis. A correlation test was performed to evaluate the association of methylation of each probe and expression of the miR-137. Furthermore, the expression changes were correlated with plaque and tangle load. All computations and statistical analyses for this study were performed using R (version 3.3.2; <https://www.r-project.org>) and RStudio (version 1.0.153)[22].

Results

DNA Methylation

Hydroxymethylation levels of all tested probes were not detectable and thus disregarded for any analysis performed (data not shown). Note that genome-wide methylation levels of the *MIR137* locus, as assessed by the BS signal, will be mentioned as methylation henceforth. Since little is known about the stability of methylation marks of microRNAs in post-mortem brain tissue, and specifically miR-137, we assessed whether PMI affected the methylation profile of *MIR137*. Across the range of PMIs used in the present study, no correlation for any of the tested probes was observed (data not shown). Therefore, age, sex and SVs (but not PMI) were used as covariates in the regression models for methylation assessment for the analysis performed. MTG *MIR137* methylation levels were compared between AD patients and unaffected controls (BSHRI cohort), while the blood methylation levels were compared between non-converting controls and converters (AgeCoDe cohort) i) at baseline (T1), ii) follow-up (T2) as well as iii) over time.

Overall, after multiple testing correction, no differentially methylated cytosines in the vicinity of *MIR137* in the MTG were identified by our analysis when comparing AD patients and age-matched controls (Figure 2). However, the methylation profile of probe cg10489614 was significantly correlated with temporal lobe plaque load ($r = 0.31$, nominal $P = 0.005$; Figure 3A) and temporal lobe neurofibrillary tangle load ($r = 0.23$, nominal $P = 0.044$; Figure 3B).

In the blood, overall, baseline methylation in the *MIR137* locus did not predict AD diagnosis and, furthermore, *MIR137* methylation status did not change with disease diagnosis. However, methylation levels for the same probe were associated with pathology in the brain (cg10489614) tended to be lower in the converter subgroup at T2, although this difference did not reach statistical significance post multiple testing correction ($p = 0.07$). Detailed methylation profiles of all tested probes can be found in Figure 4.

MTG miR-137 expression in AD

An exploratory approach was followed in order to identify the factors that alter miR-137 expression. Correlation analysis revealed that the PMI had a significant effect on expression ($r = -0.265$, $p = 0.022$; Figure 5A), and thus was used as a covariate for subsequent analysis alone with age and sex. A linear regression model was generated to assess differential miR-137 expression with disease diagnosis in the MTG. No expression changes with disease diagnosis were found (Figure 5B). Nevertheless, we identified a tendency for decreased expression with increased plaque load ($r = -0.21$, nominal $P = 0.056$; Figure 5C) and neurofibrillary tangle load ($r = -0.2$, nominal $P = 0.071$; Figure 5D). No correlation between MTG methylation and expression of miR-137 was found (data not shown).

Discussion

Two main outcomes emerge from this study. First, a specific *MIR137* locus position tended to be differentially methylated in the blood of AD patients, and further, the same probe was associated with A β plaque and neurofibrillary tangle load in the MTG. Second, miR-137 expression, but not methylation, was highly affected by the PMI, suggesting miR-137 expression profiles in post-mortem tissues should be interpreted with caution, and that methylation may be a more stable measure in this respect.

Here, we show that there is a tendency for variable methylation pattern of the *MIR137* locus in blood specimen between controls and individuals diagnosed with AD. Within the MTG of AD patients, no methylation differences were detected in the *MIR137* region studied. To date, along with this study, there is one more interrogating DNA methylation of miRNAs in AD [23]. That study followed a similar method with considerably smaller sample size ($N = 10$), conducting a targeted analysis with all probes annotated for microRNA genes in the temporal cortex and, consistent to our results, no differential methylation was reported for the *MIR137* locus.

Nevertheless, our study shows a strong association between aggravation of AD hallmarks and increased methylation of probe cg10489614 was shown. Concerning AD pathology, aberrant regulation of multiple miRNAs has been shown to affect cascades related to AD progression. MiR-29a/b-1 and miR-106 directly target amyloid precursor protein (APP) and/or beta-secretase 1 (BACE1) thus promoting A β metabolism [24, 25]. Further, miR-15, miR-26b as well as miR-125b modulate tau hyperphosphorylation [26-28]. Up to date miR-137 expression has been indirectly linked to A β [12], and this study is the first one to hint at a connection between *MIR137* methylation and AD pathology. Currently the underlying mechanism of action remains uninvestigated. Differential methylation of *MIR137* locus could induce changes to the binding affinity of transcription factors on adjacent promoters. More specifically, a newly characterized promoter located within the *MIR137* locus at chr1:98511662-98511917 is a binding site for repressor element-1 silencing transcription factor (REST) [29], a gene reported to have neuroprotective properties, via repressing pro-apoptotic genes [30]. In relation to AD, REST is highly dysregulated in the early stages of the disease, making neurons more vulnerable to oxidative stress, A β toxicity and hence leading to degeneration [30]. Therefore, it can be hypothesized that the reported correlation between methylation levels and hallmark pathology could be mediated by differential binding of REST onto the *MIR137*-neighboring promoter thus hindering the neuroprotective action of REST and promoting AD-related pathology. Further research on the link between methylation and pathology is vital in order to test this hypothesis and elucidate the exact mechanism of action.

In this study we indicate that while there is a tendency for decreased methylation in the *MIR137* locus peripherally, this modification does not follow the same pattern centrally. In the brain the differential methylation of the same position is more closely associated with aggravated disease hallmark manifestation. At this point it should be mentioned that one should not -necessarily- expect to notice comparable methylation fluctuations in brain and blood specimens. Indeed, using the Blood Brain DNA Methylation Comparison Tool [31] we can check the expected methylation pattern in blood and brain of the five studied probes under investigation. Since this online tool provides information for 4 specific brain areas (PFC, entorhinal cortex, superior temporal gyrus and cerebellum), we considered the superior temporal gyrus as the most spatially relevant structure to the MTG for further analysis. According to the tool, no significant correlation between brain and blood methylation patterns is to be expected in any of the tested probes (Supplementary Table 2). Regarding the significant correlation of the MTG methylation with the aggravation of the neuropathology, one should consider that quantitative measurements of pathological hallmarks might be more accurate and more representative of disease status than the case-control binary classification system. Here in order to compare the blood-brain methylation patterns we used the same analysis parameters, considering AD diagnosis as the

predictor, possibly disregarding that some individuals did not reach the definite criteria for diagnosis but have signs of rather advanced pathology.

Previously, Geekiyanage et al. showed significantly downregulated miR-137 levels in the cortex and serum from AD patients, which they associated with an increase in β -amyloid levels via an increase in serine palmitoyltransferase [12, 13]. Additionally, decreased levels of miR-137 induced by chromosomal microdeletion of 1p21.3, the locus that hosts *MIR137HG*, have been associated with intellectual disability, a phenotype that is characterized by cognitive impairment [32]. The latter observation is vastly associated with the symptomatology of AD, rather than the pathology per se. Our results do not show differential miR-137 expression in AD, but they do indicate a tendency for decreased expression with the aggravation of AD pathology, as interpreted by amyloid plaque and neurofibrillary tangle load. Here, the tissue available for miR-137 quantification was post-mortem material. While the general consensus in literature is that miRNAs are stable molecules and have lower decay rates in post-mortem tissues [33], we show that the PMI, as low as it is in the current study, highly affects the detected expression of miR-137. To date, the turnover profile of miR-137 has not been identified but it has been speculated in literature that its half-life is rather short [34]. Interlacing our observations with this hypothesis, we suggest that variation in PMI influences the decay rate of the molecule and hence quantification of expression in post-mortem material should be performed and interpreted with caution. Instead, for a more stable measurement we suggest using the methylation levels of *MIR137*, which is not affected by this variable.

Overall, in this study, subtle methylation and expression changes were detected in the MTG of AD patients. The specific structure is not yet well-characterized with respect to AD progression. A recent study by Ray and Zhang showed that the MTG is less severely affected in AD than the hippocampus and the entorhinal cortex and, thus, they advocate that its pathological status is more indicative of early AD pathology [35]. Indeed here we demonstrate that aggravation of AD-related pathology in the temporal lobe, a brain area highly infested by plaques and tangles can capture these differences in methylation and expression more efficiently. Therefore, it could be speculated that while studying the MTG contributes highly to the collective knowledge about AD, it might not be the most characteristic structure to unveil AD-associated methylation and expression profiles of miR-137. It is tempting to speculate that more distinctive patterns and higher variation in miR-137 expression and methylation between AD and controls will be observed in the hippocampus, a structure severely affected by disease pathology and more importantly, a structure which cellular physiology is highly regulated by miR-137, namely neurogenesis and neuronal differentiation [36]. Thus it would be of high interest to co-examine the hippocampal methylomic and transcriptomic profile of miR-137 as part of a perspective study.

Recent research lines have started elucidating the role of miR-137 in the pathophysiology and symptomatology of AD, focusing primarily on the expression levels of the miRNA in a binary manner, control vs. AD. In the same vein as the reported approaches, this study examined MTG and blood methylation as well as MTG expression changes of miR-137, and overall did not show any differences when comparing AD and unaffected controls. However, we do demonstrate a strong correlation of increased methylation of a single probe and decreased expression of miR-137 with aggravation of AD-related pathology. This outcome suggests that transcriptional-epigenetic mechanisms within the *MIR137* locus are associated with AD hallmarks, either as markers of the disease progression or as causal factors of the hallmark accumulation. To our knowledge, this is the first study to expand this research line beyond the genomic level and interrogate the methylomic profile of *MIR137* in AD. Complementary research lines employing cohorts with higher sample sizes should be conducted in order to define the exact role of miR-137 methylation in AD and unveil additional molecular mechanisms that might mediate its effect on AD pathology. Further, our results prompt for careful consideration of covariates, like PMI, that might influence the decay of the mRNA molecules leading to false positive or false negative results.

In conclusion, this study shows subtle shift towards increased site-methylation and decreased expression of the *MIR137* within the MTG with aggravation of the pathological hallmarks of AD. Additionally, the same probe exhibits decreased methylation levels in blood with disease diagnosis. The reported results, while not profound, they hint towards an involvement of miR-137 and its methylation state in the progression of AD and thus spur for an in-depth interrogation of the biological repercussions this mechanism could have. Perspectively, comparable studies in brain structures more closely related with disease pathology as well as the biological function of miR-137, namely the hippocampus and the frontal cortex, could be conducted. To this end not only would it be possible to globally characterize the profile of the specific miRNA but also ultimately assess whether it has a causal or resulting role in the aggravation of the pathological hallmarks of AD.

Figure 1: Genomic location of the MIR137 locus and relevant positions of the probes under examination in this study (adjusted from Genome Data Viewer, NCBI).

BSHRI cohort		
N	35	45
Sex (male/ female)	17/18	22/23
Age at death (Mean $\pm$ SD)	84.46 $\pm$ 5.50	85.09 $\pm$ 6.24
PMI (Mean $\pm$ SD)	2.87 $\pm$ 1.03	2.77 $\pm$ 0.69
Braak stage (range(Median))	I - IV (III)	II - VI (V)
Plaque total (Mean $\pm$ SD)	4.65 $\pm$ 4.30	12.97 $\pm$ 2.25
Tangle total (Mean $\pm$ SD)	3.96 $\pm$ 2.10	11.02 $\pm$ 4.16
AgeCoDe cohort		
Baseline (T1)		
N	42	55
Sex (male/ female)	10/32	17/18
Age at baseline (Mean $\pm$ SD)	81.0 $\pm$ 3.11	82.33 $\pm$ 3.52
Follow-up (T2)		
N	42	42
Sex (male/ female)	10/32	13/29
Age at baseline (Mean $\pm$ SD)	81.0 $\pm$ 3.11	82.04 $\pm$ 3.47

Table 1: Demographic information of the Banner Sun Health Research Institute (BSHRI) and the German Study on Ageing, Cognition and Dementia in Primary Care Patients (AgeCoDe) cohorts. Middle temporal gyrus tissue from AD patients and non-demented controls was obtained from BSHRI and in the table are presented the sample size, the distribution of sex, age, postmortem interval (PMI; hours), Braak stage score and total plaque and tangle load, i.e. the sum of average amyloid- β plaque and tangle densities, respectively, in the entorhinal cortex, hippocampus, parietal lobe, temporal lobe and frontal lobe cortex). Blood samples from controls, who showed no signs at baseline or follow-up (controls), and converters who showed no signs of dementia at baseline, but were diagnosed with AD dementia at follow-up (AD patients) were obtained from AgeCoDe. DNA samples were collected at baseline and follow-up for both groups. Displayed is the number of samples in each group, and the distributions of sex and age at baseline.

Figure 2: MTG methylation changes of the five probes annotated for the MIR137 gene between AD patients and controls. The y – axis represents beta values regressed for age, sex and 5 surrogate variables.

Figure 3: MIR137 (cg10489614) methylation changes within the MTG with plaque (A) and tangle (B) load aggravation.

Figure 4: Blood methylation changes of the five probes annotated for the MIR137 gene between AD patients and controls overtime. The y – axis represents beta values regressed for age, sex and 5 surrogate variables.

Figure 5: (A) Correlation between miR-137 expression and post mortem interval in the BSHRI cohort. (B) miR-137 expression changes within the MTG between AD patients and controls. (C-D) miR-137 expression changes within the MTG with plaque and tangle load aggravation, respectively.

Blood-STG Methylation Comparison		
	r	p-value
cg04293733	0.14	0.22
cg05423529	0.13	0.26
cg10489614	0.24	0.06
cg14783814	0.18	0.13
cg22333214	0.17	0.15

Table 2: Correlation between blood and superior temporal gyrus (STG) methylation changes on probes annotated for the MIR137

References

1. Parodi, F., et al., Epigenetic dysregulation in neuroblastoma: A tale of miRNAs and DNA methylation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 2016. 1859(12): p. 1502-1514.
2. Martinez, B. and P.V. Peplow, Blood microRNAs as potential diagnostic and prognostic markers in cerebral ischemic injury. *Neural Regen Res*, 2016. 11(9): p. 1375-1378.
3. Olde Loohuis, N.F.M., et al., The schizophrenia risk gene MIR137 acts as a hippocampal gene network node orchestrating the expression of genes relevant to nervous system development and function. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2017. 73: p. 109-118.
4. Lardenoije, R., et al., The epigenetics of aging and neurodegeneration. *Prog Neurobiol*, 2015. 131: p. 21-64.
5. Van den Hove, D.L., et al., Epigenetically regulated microRNAs in Alzheimer's disease. *Neurobiol Aging*, 2014. 35(4): p. 731-45.
6. Sempere, L.F., et al., Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome biology*, 2004. 5(3): p. 1.
7. Yin, J., et al., miR-137: a new player in schizophrenia. *International journal of molecular sciences*, 2014. 15(2): p. 3262-3271.
8. Sun, J., et al., MiR-137 inhibits proliferation and angiogenesis of human glioblastoma cells by targeting EZH2. *Journal of neuro-oncology*, 2015. 122(3): p. 481-489.
9. Kunej, T., et al., Cross talk between microRNA and coding cancer genes. *Cancer journal (Sudbury, Mass.)*, 2012. 18(3): p. 223.
10. Cosgrove, D., et al., MiR-137-derived polygenic risk: effects on cognitive performance in patients with schizophrenia and controls. *Transl Psychiatry*, 2017. 7: p. e1012.
11. Willemsen, M.H., et al., Chromosome 1p21.3 microdeletions comprising DPYD and MIR137 are associated with intellectual disability. *J Med Genet*, 2011. 48: p. 810-818.
12. Geekiyanage, H. and C. Chan, MicroRNA-137/181c regulates serine palmitoyltransferase and in turn amyloid β , novel targets in sporadic Alzheimer's disease. *The Journal of Neuroscience*, 2011. 31(41): p. 14820-14830.

13. Geekiyanage, H., et al., Blood serum miRNA: Non-invasive biomarkers for Alzheimer's disease. *Experimental Neurology*, 2012. 235(2): p. 491-496.
14. Luck, T., et al., Risk factors for incident mild cognitive impairment—results from the German Study on Ageing, Cognition and Dementia in Primary Care Patients (AgeCoDe). *Acta Psychiatrica Scandinavica*, 2010. 121(4): p. 260-272.
15. Braak, H. and E. Braak, Neuropathological staging of Alzheimer-related changes. *Acta neuropathologica*, 1991. 82(4): p. 239-259.
16. Luck, T., et al., Mild cognitive impairment in general practice: age-specific prevalence and correlate results from the German study on ageing, cognition and dementia in primary care patients (AgeCoDe). *Dementia and geriatric cognitive disorders*, 2007. 24(4): p. 307-316.
17. Ramirez, A., et al., Elevated HbA 1c is Associated with Increased Risk of Incident Dementia in Primary Care Patients. *Journal of Alzheimer's Disease*, 2015. 44(4): p. 1203-1212.
18. Schalkwyk, L., et al., watermelon: Illumina 450 methylation array normalization and metrics. R package version, 2013. 1(2).
19. Leek, J.T. and J.D. Storey, Capturing Heterogeneity in Gene Expression Studies by Surrogate Variable Analysis. *PLOS Genetics*, 2007. 3(9): p. e161.
20. Pinheiro J, B.D., DebRoy S, Sarkar D and R Core Team, nlme: Linear and Nonlinear Mixed Effects Models. 2017.
21. Du, P., W.A. Kibbe, and S.M. Lin, lumi: a pipeline for processing Illumina microarray. *Bioinformatics*, 2008. 24(13): p. 1547-1548.
22. Team, R.C., R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013. 2014.
23. Villela, D., et al., Differential DNA Methylation of MicroRNA Genes in Temporal Cortex from Alzheimer's Disease Individuals. *Neural Plasticity*, 2016. 2016: p. 2584940.
24. Hébert, S.S. and B. De Strooper, Alterations of the microRNA network cause neurodegenerative disease. *Trends in neurosciences*, 2009. 32(4): p. 199-206.

25. Hébert, S.S., et al., Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/ β -secretase expression. *Proceedings of the National Academy of Sciences*, 2008. 105(17): p. 6415-6420.
26. Banzhaf-Strathmann, J., et al., MicroRNA-125b induces tau hyperphosphorylation and cognitive deficits in Alzheimer's disease. *The EMBO Journal*, 2014. 33(15): p. 1667-1680.
27. Hébert, S.S., et al., Genetic ablation of Dicer in adult forebrain neurons results in abnormal tau hyperphosphorylation and neurodegeneration. *Human molecular genetics*, 2010. 19(20): p. 3959-3969.
28. Absalon, S., et al., MiR-26b, upregulated in Alzheimer's disease, activates cell cycle entry, tau-phosphorylation, and apoptosis in postmitotic neurons. *Journal of Neuroscience*, 2013. 33(37): p. 14645-14659.
29. Warburton, A., et al., Characterization of a REST-regulated internal promoter in the schizophrenia genome-wide associated gene MIR137. *Schizophrenia bulletin*, 2014. 41(3): p. 698-707.
30. Lu, T., et al., REST and Stress Resistance in Aging and Alzheimer's Disease. *Nature*, 2014. 507(7493): p. 448-454.
31. Eilis Hannon, K.L., Leonard Schalkwyk , Jonathan Mill. Blood Brain DNA Methylation Comparison Tool. Available from: <http://epigenetics.essex.ac.uk/bloodbrain/>.
32. Gotz, J., et al., Formation of neurofibrillary tangles in P301 τ transgenic mice induced by A β 42 fibrils. *Science*, 2001. 293.
33. Price, J.L. and J.C. Morris, Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. *Ann Neurol*, 1999. 45.
34. Hardy, J. and D.J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*, 2002. 297.
35. Ray, M. and W. Zhang, Analysis of Alzheimer's disease severity across brain regions by topological analysis of gene co-expression networks. *BMC Systems Biology*, 2010. 4: p. 136-136.
36. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. *Nature medicine*, 1998. 4(11): p. 1313-1317.

5 On the epigenetic theory of major psychosis: schizophrenia- and bipolar disorder-associated methylomic signatures in the cerebellum

Abstract	p.135
Keywords	p.135
Introduction	p.136
Methods	p.138
Sample Information	p.138
DNA isolation and sodium bisulfite treatment	p.138
Genome-wide DNA methylation analysis	p.138
Quality control (QC) and data normalization	p.139
Statistical analysis	p.140
Surrogate variable analysis	p.140
Regression model and differentially methylated positions	p.140
Differentially methylated regions	p.140
Results	p.141
Discovery stage - SMRI Array Collection	p.141
Replication stage - SMRI Neuropathology Consortium	p.142
Shared DMPs and DMRs	p.143
Discussion	p.144
References	p.166

Artemis Iatrou¹, Emma L. Dempster,² Eilis Hannon², Ehsan Pishva^{1,2}, Roy Lardenoije¹, Joe Burrage², Bart Rutten¹, Gunter Kenis¹, Daniel L.A. van den Hove^{1,3,4}, Jonathan Mill^{2,4}

1 School for Mental Health and Neuroscience (MHeNs), Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, the Netherlands

2 University of Exeter Medical School, University of Exeter, Exeter, UK

3 Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Wuerzburg, Germany

4 Equal contribution to this work

Abstract

Intensive research on the aetiopathogenesis of disorders within the major psychosis spectrum, namely schizophrenia (SZ) and bipolar disorder (BP) has highlighted a critical role of epigenetic processes in the development of these disorders. Furthermore contemporary approaches for a global understanding of the pathophysiology of major psychosis have provided sufficient clinical and experimental evidence to support an important role of the otherwise overlooked cerebellum in psychosis-related cognitive and emotional dysfunctional networks. Building on these research lines, this study aimed to identify cerebellar DNA methylation signatures associated with major psychosis phenotypes by following an epigenome-wide approach. Post mortem cerebellar tissue from patients diagnosed with SZ, BP or controls was used in order to identify psychosis or disease-specific methylomic signatures, by means of the Illumina Infinium MethylationEPIC BeadChip (EPIC) array. While no collective cytosine methylation changes were observed, two regions at the ZFP57 and PM20D1 loci were significantly hypermethylated in both SZ and BP. The region associated with ZFP57 emerged as a BP-related methylation signature in an independent replication cohort. Our study represents the first analysis of epigenetic variation associated with major psychosis leveraging the EPIC technology. To this end, it highlights that the BP-afflicted cerebellum is imprinted with distinct methylation signatures, a finding consistent between two independent cohorts.

Keywords: Major psychosis; Schizophrenia; Bipolar Disorder; DNA methylation; Cerebellum

Introduction

Mental disorders are characterized by severe distress, impaired personal functioning and behavioural abnormalities, that may persist and significantly impact the quality of life of the affected individual. Schizophrenia (SZ) and bipolar disorder (BP), while on the one hand considered separate pathological entities with a differential aetiopathogenesis, are also both categorized under the umbrella term major psychotic disorders, with a partially shared underlying aetiology [1, 2]. SZ, or psychosis spectrum disorder [3], affecting people in their late adolescence or young adulthood, is characterized by delusions and hallucinations as well as disturbed emotional responses, reasoning and concentration [4]. BP, is characterized by a constellation of symptoms that interchange from episodes of mania or hypomania to periods of depression [4].

The aetiology of major psychosis still remains rather elusive, regardless the increasing body of research dedicated to unravel it. While highly heritable (70-90% for SZ and 60-80% for BP)[5], common genetic variants fail to account for disease aetiology, and at the same time, rare genetic variants cannot sufficiently explain the attributable risk [6]. Yet again, disease-discordant monozygotic twin percentages have been reported as high as 41-65% for SZ and 60% for BP [7, 8], which cannot be solely explained by environmental influences. Thus, the prevailing theory for interpreting disease causality endorses a genetic diathesis combined with in-utero and/or childhood and adolescent exposure to environmental risk factors. Contemporary notions of an epigenetic involvement are becoming all the more popular, since this mechanism could not only bridge gene x environment interactions but also affect gene expression and structure in a stochastic manner. To this end, the role of epigenetic mechanisms as susceptibility mediators has been interrogated in both SZ and BP. Epigenome-wide association studies (EWAS) have profiled the methylome of blood [9-11] as well as frontal cortex [2, 12, 13], striatum [13], hippocampus [13], anterior cingulate [12] and cerebellum [14] from patients with either or both disorders. Furthermore, cross-tissue studies have highlighted that DNA methylation alterations highly depend on distinct brain regions [12, 13].

Although overlooked for a long time, recently, an important role has been attributed to the cerebellum with respect to neuropsychiatric disorders [15]. Anatomically, reduced cerebellar volume has been reported as a trait-related characteristic in BP [16]. Functionally, reduced activation has been associated with major psychosis [17-20] and dysfunctional cortico-thalamic-cerebellar connections have been connected to cognition-related networks in SZ [21]. Complementary to the latter, there is increasing evidence of the role of the cerebellum as a regulator

of emotional and cognitive responses, traits impaired in both disorders [15, 22, 23]. At a molecular level, differential cerebellar expression of N-methyl-D-aspartate (NMDA) receptor subunits and reduced γ -aminobutyric acid (GABA) A and GABAB expression have been observed in patients with SZ [24] and major psychotic disorders [25, 26], respectively. Moreover, the expression of various genes encoding neuronal ion-channel subunits is downregulated in the cerebellum of SZ patients [27]. Finally, a genome-wide expression and methylation study performed on cerebellum samples from individuals with major psychosis identified numerous differentially expressed genes (*PIK3R1*, *BTN3A3*, *NHLH1*, and *SLC16A7*), concomitant with deviant methylation patterns within the cis region of these genetic loci [14]. Nevertheless, no explicit methylomic signatures were associated with SZ or BP or major psychosis.

In the present study, we examined psychosis-specific cerebellar methylation signatures in two cohorts by conducting two independent EWAS. Furthermore, we endeavored to disentangle the general effects of the major psychosis phenotype and attribute them to either SZ or BP. To this end, three analysis models were generated in order to profile the methylome of SZ, BP as well as major psychosis. *Post-mortem* cerebellar tissue from the Array Collection was used as the discovery sample, whereas comparable tissue samples from the Neuropathology Consortium served as a replication sample. Our study is unique as it is the first to use the Illumina Infinium MethylationEPIC BeadChip (EPIC array) technology in order to profile the methylation status of more than 850K CpGs across the genome of the cerebellum in major psychosis. Overall, no global methylation differences were observed when comparing controls and any of the three analyses performed. However, we demonstrate that there are two regions at the *ZFP57* and *PM20D1* loci that are significantly hypermethylated in both SZ and BP. Most importantly, a region associated with *ZFP57* emerged as a significant methylation signature for BP both in the discovery and replication cohort. All in all, our data indicate that distinct neuropsychiatric-related epigenetic modifications are imprinted in the afflicted cerebellum and reinforce further research towards testing the hypothesis that epigenetic changes play an important role in the pathophysiology of neuropsychiatric disorders.

Methods

Sample Information

Cerebellum tissue from two different cohorts, the Array Collection and the Neuropathology Consortium, was provided by the Stanley Medical Research Institute (SMRI) [28]. The Array Collection consists of post-mortem material of 35 individuals in each of three diagnostic groups: SZ, BP and unaffected controls, and for this study, cerebellum tissue from 95 samples was used (see Table 1 for details). The cerebellum tissue from the Neuropathology Consortium used in this study consisted of 15 SZ, 15 BP and 15 unaffected controls. These specimens were collected, with informed consent from next of kin, by participating medical examiners, between January 1995 and June 2002. Additional information on inclusion and exclusion criteria can be found at the original publication of the SMRI [28]. Detailed demographic information can be found in Table 1.

DNA isolation and sodium bisulfite treatment

Genomic DNA (gDNA) from both cohorts was isolated by overnight digestion of approximately 100 mg fresh-frozen cerebellum tissue at 55°C using cell lysis buffer (100 mM Tris.HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and fresh proteinase K (20 µg/µL), followed by RNase A (100 mg/mL, Qiagen) treatment and DNA extraction using a phenol, chloroform, isoamyl and ethanol mixture. After precipitation and washing with ethanol, samples were resuspended in 50 µL TE buffer (pH 8.0) and stored at -20°C. From the samples that passed quality control, 500 ng DNA was treated with sodium bisulfite (BS) using the Zymo EZ-96 DNA methylation kit (Zymo Research, CA, USA) according to the manufacturer's protocol.

Genome-wide DNA methylation analysis

BS-treated DNA from the discovery cohort (SMRI Array Collection) and the replication cohort (SMRI Neuropathology Consortium) was assessed using the Illumina Infinium MethylationEPIC BeadChip (850K; EPIC) array and Illumina Infinium 450K Methylation BeadChip (450K) array (Illumina, Inc., CA, USA), respectively, and was further quantified using an Illumina HiScan System (Illumina, CA, USA). All samples were assigned a unique code for the purpose of the randomized with respect to sex and disease status to avoid batch effects. Illumina Genome Studio software was used to extract the raw signal intensities of each probe (without background correction or normalization).

Quality control (QC) and data normalization

Raw signal intensities extracted with Illumina Genome Studio were imported into the R statistical environment with function from the methylumi package [29] and further processed using the watermelon package [30]. The pipeline for quality control (QC) was consistent for both array types, unless stated otherwise. Initial QC assessment was performed using functions in the methylumi package to check concordance between reported and actual gender, based on interrogation of the sex chromosomes. From the discovery cohort, one sample was dropped out as it displayed inefficient BS conversion, identified with function in the watermelon package, ($\leq 85\%$) and one additional according to the guidelines of SMRI. Moreover, two more had to be dropped due to gender mismatch, thus resulting in 91 samples. No samples were dropped out from the replication cohort. Further preprocessing was conducted using the watermelon package by applying a p-filter [30]. Probes having more than 1% of samples with a detection p-value greater than 0.05 and a beadcount lower than 3 in more than 5% of samples. Finally, the filtered data were normalized with dasen [30]. Non-CpG Single nucleotide polymorphism (SNP) probes, probes that had been reported to contain common (MAF $>5\%$) SNPs in the CG or single base extension position, or probes that were non-specific or mismapped [31, 32], were flagged and disregarded in the evaluation of our results. Additionally, from the EPIC dataset, probes flagged by Illumina were also disregarded. The resulting datasets for further analysis consisted of 91 samples and 836,055 probes for the discovery cohort, and, 45 samples and 420,115 probes for the replication cohort. The distribution of beta values from each dataset can be found in Supplementary Figure 1.

Statistical analysis

For downstream statistical analysis, three different models were considered for both cohorts: one with the two disorders pooled together and hence distinguishing between psychiatric and non-psychiatric/control phenotypes (PSY), one for the bipolar disorder versus controls (BP) and one for the schizophrenia patients versus controls (SZ).

Surrogate variable analysis

Surrogate variable (SV) analysis (SVA) was performed in order to determine and estimate variation stemming from unknown sources, a common phenomenon in high-throughput experiments [22]. For each dataset, 5 SVs were identified and were then correlated with variables known to affect methylation status, as well as lifestyle variables provided by the SMRI. SVs that explained most of the variation were selected to be included in subsequent analyses as covariates.

Regression model and differentially methylated positions

Differentially methylated cytosine positions (DMPs) associated with major psychosis phenotype or disease diagnosis were identified. A linear regression analysis was performed with the beta values from each of the three subsets (PSY, BP, SZ) as outcome and psychiatric phenotype or disorder diagnosis as predictor, respectively. Five SVs from each dataset were included as covariates in the model along with age, sex and batch effect as defined by chip barcode. The inflation rate of the generated model was assessed based on the lambda (λ) value and the respective QQ plot generated with function from the qqman package [33]. P-values were adjusted for multiple testing using the Benjamin-Hochberg false discovery rate (FDR) procedure with functions from the qvalue package [34]. Probes were ordered based on a combined p-value and regression estimate/coefficients ranking, and then the highest ranked DMPs were selected (Table 2-4). The Manhattan and QQ plots for the three analyses from each of the two cohorts can be found in Figure 1 and 2, respectively. Illumina EPIC and 450K array probes were annotated using the Illumina UCSC gene annotation.

Differentially methylated regions

In order to identify differentially methylated regions (DMRs), the Python module comb-p was used [35]. With this package, p-values smaller than 0.001 are correlated in order to detect groups of more than 3 spatially correlated CpGs within a 1000 base pair sliding window across the genome. Obtained p-values were Stouffer-Liptak-Kechris (SLK) corrected for adjacent p-values, and were subsequently corrected for multiple testing using the Šidák correction.

Gene ontology (GO) enrichment analysis was conducted using the missMethyl package (version 1.8.0) [36], which takes into account potential bias due to different number of probes for each gene. Probes with an uncorrected $p \leq 0.001$ were used ($N_{\text{PSY}} = 858$, $N_{\text{BP}} = 753$, $N_{\text{SZ}} = 897$) enrichment analysis, whereas probes included in the regression analysis were used as background lists. The highest ranked GO terms can be found in Supplementary Table 1-3.

Results

Discovery stage - SMRI Array Collection

For the discovery stage of our analysis, we used cerebellar tissue from 105 individuals from the SMRI Array collection, equally distributed over 3 conditions: bipolar disorder (BP, $n = 35$), schizophrenia (SZ, $n = 35$) and controls (CTR, $n = 35$). The core of the analysis applied here was to identify DMPs and DMRs in major psychosis (PSY) associated either with BP or SZ.

Focusing on PSY, 5.08% of the tested probes (42,438 probes) passed nominal significance levels (uncorrected $p \leq 0.05$) but neither of them reached statistical significance after FDR correction. Among the top-ranked DMPs (Table 2), probes annotating to non-coding genes and genes related to neuronal development can be found (Table 2). Worth mentioning are probes cg01684722 (#9, nominal $P = 1.72\text{E-}05$, FDR p value = 0.9) and cg21865845 (#29, nominal $P = 3.71\text{E-}05$, FDR p value = 0.9) that are annotated to *TIAM2* and *ROBO1*, respectively. SNPs in both genes have been previously associated with schizophrenia [37, 38]. Additionally, five DMRs surviving multiple testing correction were identified (Table 5), with the one on *ZFP57* including 20 probes (nominal $P = 2.49\text{E-}14$, Šidák $p = 2.81\text{E-}11$). Additionally, the *PTPRF* gene was top-ranked in both the DMP and DMR analysis. Notably, all detected DMRs displayed hypermethylation in cases when compared to controls.

When analyzing the results based for each disorder separately, 4.7% and 5.2% of the probes for BP and SZ, respectively, passed nominal, but not FDR-adjusted significance levels (Table 3-4). Among the nominally significant probes, 7,190 were shared between the two disorders. The top-ranked BP- and SZ-associated DMPs are shown in Tables 3 and Table 4, respectively. Interesting “hits” detected in BP were probes in the vicinity of *GNB1L*, a candidate gene for BP and SZ [39], and *KIF3*, a gene expressing an interactive protein of DISC1 [40], a usual suspect in major psychosis [41]. Similarly, noteworthy SZ-associated DMPs were located within the *KCNQ1* gene, which is associated with elevated risk for mental disorders [42], and *CACNA2D4*, where deletions have been associated with

psychiatric phenotypes [43]. The DMR analysis revealed 3 BP-associated and 7 SZ-associated regions that survived correction for multiple testing (Table 5). The highest ranked one in both datasets was *PM20D1*, while *ZFP57* was also affected in both disorders. These DMRs were all hypermethylated in cases versus controls.

Concerning the GO-term enrichment analysis on the SZ subset, two terms were significantly enriched post-FDR correction (GO:0007156; P nominal = 2.27E-08, FDR p value = 1.8E-04; GO:0098742; P nominal = 1.84E-07, FDR p value = 7.28E-04). These SZ-enriched terms are both related to cell-cell adhesion biological processes, which involve cadherin-related genes. Interestingly, both these terms have been recently associated with pathological features of schizophrenia and psychosis [44-46].

Replication stage - SMRI Neuropathology Consortium

An independent EWAS was conducted on the SMRI Neuropathology (NP) Consortium cohort which consisted of cerebellar tissue from 45 individuals, equally distributed over 3 conditions: bipolar disorder (BP, n = 15), schizophrenia (SZ, n = 15) and controls (CTR, n = 15). A similar analytical line as the aforementioned in the discovery cohort was followed: first we considered the existence of a psychiatric phenotype as predictor and then we studied each disorder separately.

For the first approach, 24,977 probes (5.95%) emerged as nominally significant. Among them (Table 6), two probes (cg20483962, cg01221018) reached statistical significance post FDR correction (cg20483962: effect size β = -0.013, nominal P = 1.26E-07, FDR p value = 0.04; cg01221018: effect size β = -0.038, nominal P = 1.93E-07, FDR p value = 0.04). Probe cg20483962 is located on chromosome 17, 215 bp upstream the transcription start site of *DVL2* (Dishevelled Segment Polarity Protein 2), which encodes a protein that acts as intracellular mediator of Wnt signalling pathway [47]. Notably, reduced expression of *DVL2* has been associated with the severity of negative symptoms in psychosis [48]. The second probe, cg01221018, is located on chromosome 14, 606 bp downstream of the transcription start site of *MDP1* (magnesium-dependent phosphatase-1). The top highest ranked 30 methylated loci can be found in Table 6 and the methylation changes of the two highest ranked probes when comparing major psychosis and unaffected individuals are depicted in Figure 3. Additionally, 5 DMRs were identified to be associated with psychiatric phenotype (Table 9). Among them was a region consisting of 7 CpGs, annotated for *BAIAP2* (brain-specific angiogenesis inhibitor 1-associated protein 2) gene, (nominal P = 4.30E-08, Šidák p = 2.90E-05). *BAIAP2* encodes an adaptor protein, involved in membrane and actin dynamics in dendritic spines [49], and rare mutations within

the locus have been associated with SZ [49-51]. Recently, a study focusing on the mediotemporal gyrus showed that methylation in this specific gene was also associated with SZ-related pathology [52]. There, following the same approach, two probes within *BAIAP2* gene body (cg01276536, cg23261327) were detected hypomethylated, suggesting a region specific, yet important role for this genetic locus.

Focusing on BP, 4.5% of the tested probes displayed nominal significant differential methylation, with no probes reaching FDR-adjusted significance levels (Table 7-8). Similarly, no SZ-associated probes passed FDR-adjusted significance levels, but 5.7% of the tested probes were nominally significant. Top 30 BP and SZ-associated DMPs are presented in Table 7 and 8, respectively. Among the nominally significant probes, the two disorders shared 944 out of 18,990 differentially methylated positions. When assessing regions displaying differential methylation, 2 BP-associated and 12 SZ-associated DMRs were identified (Table 9).

Shared DMPs and DMRs

We proceeded with correlating the uncorrected beta values from the common probes of the discovery and the replication cohort that passed nominal significance levels (uncorrected $p \leq 0.05$). The two datasets shared 1,114 psychiatric phenotype-associated probes, 885 BP-associated probes and 1,125 SZ-associated probes out of the entirety of the probes interrogated by the EPIC array. Among them the ones with the most significant beta values (uncorrected $p \leq 0.01$) were selected for subsequent correlation analysis. No correlation was detected between the probes associated with psychiatric phenotype ($n = 44$, $r = 0.18$, $p = 0.24$) and BP ($n = 27$, $r = 0.11$, $p = 0.57$). However, SZ-associated DNA methylation scores for the 59 shared most significant probes from the two datasets were significantly correlated ($n = 59$, $r = 0.26$, $p = 0.046$; Figure 4).

Focusing on the DMR analysis, the BP-associated region annotating the *ZFP57* gene that emerged from the discovery cohort was also replicated by the replication cohort. More specifically, in the discovery cohort, an intergenic region located on chromosome 16 spanning from 29,648,345 bp to 29,648,605 bp contained 12 hypermethylated CpGs (nominal $P = 8.74E-07$, Šidák $p = 0.00028$), an observation that was reproduced in the replication cohort (16: 29,648,161-29,648,757, 18 hypermethylated probes, nominal $P = 3.78e-26$, Šidák $p = 2.664e-23$).

Discussion

This is the first EWAS conducted on the cerebellum of patients with bipolar disorder and schizophrenia, using the EPIC array. Our approach aimed to study these disorders in conjunction as well as separately in order to identify both mutual and disease-specific epigenetic signatures. To this end, we demonstrate a rather distinctive methylome between the disorders with few common “hits”. More importantly, we provide evidence for cerebellum-specific hypermethylation within the *ZFP57* gene, associated with both major psychosis in general, but more specifically in BP.

The DMR located in the *ZFP57* gene was significantly more methylated in psychotic patients, in particular patients suffering from BP. It is of high importance that the BP-associated *ZFP57* hypermethylation was additionally identified by an independent EWAS conducted on the replication cohort. *ZFP57* encodes a zinc finger protein preferentially expressed early in development [53, 54] and downregulated upon differentiation, yet with detectable levels at oligodendrocytes [54]. The *ZFP57* protein is of particular importance as it is crucial for survival during the embryonic stage. Interestingly, *ZFP57* is closely entwined with the epigenetic machinery as it is auxiliary to the maintenance of both maternal and paternal imprinting marks during development by indirectly targeting DNA methyltransferases [55]. Hence, *ZFP57* plays a very important role in human development. Additionally it is a repressor of activated transcription and is mainly associated with repression of the promoter regions of Schwann cell myelin-associated genes [54]. This is directly relevant to the increasing scientific evidence supporting the involvement of epigenetically-dysregulated glial cells to the pathogenesis of neuropsychiatric disorders [56]. Therefore, functionality-wise, aberrant *ZFP57* methylation and/or dysregulated expression could be associated with the neurodevelopment aspects of psychosis and contribute to its pathogenesis.

The past years, the methylomic profile of *ZFP57* has been the centre of attention in studies focusing on psychiatric and neurodevelopmental disorders. Most recently, Rivollier et al. indicated hypermethylation at exactly the same DMR that we report here in the blood of individuals with psychosis, mainly schizophrenia, that were exposed to diethylstilbestrol [57]. This observation is in accordance with our hypermethylated SZ-associated DMR in the *ZFP57* gene. Furthermore, Ladd-Acosta et al. described a DMR within the *ZFP57* gene, which includes the probes reported here, to be significantly more methylated in the temporal cortex of patients with autism spectrum disorder [58]. Although two distinct disorders, autism and schizophrenia bare multiple common phenotypic traits, and, as they

co-occur, it has been speculated that they share pathological features of deviant neurodevelopment and plasticity [59]. Finally, Rutten et al. reported a similar DMR linked to post-traumatic stress disorder (PTSD) [60]. Altogether, based on our results and evidence from the literature, differential *ZFP57* methylation seems to play a role in psychiatric disorders, what once elucidated will provide more insight in the pathogenesis of the disorders. We speculate that since *ZFP57* is a transcriptional regulator, it acts as a hub protein and, thus, methylomic changes within its locus may cause a chain reaction of transcriptional changes in neurodevelopmental regulatory genes, contributing to the pathogenesis of these diseases.

To date, many research lines have focused on elucidating the methylomic profile of various brain structures as well as peripheral tissue, in relation to major psychosis, SZ and, to a lesser extent, BP [2, 12, 61-63]. Yet, the cerebellum remains a highly under-investigated structure, regardless increasing evidence of its involvement in all aforementioned disorders, with only one successful EWAS conducted using Illumina Infinium HumanMethylation27 arrays, which have a substantially smaller coverage (27k probes) [14]. In this study, nominally significant probes with differential methylation patterns were detected in much smaller percentages to the ones reported here (7.74% in PSY vs. CTR; 2.35% in SZ vs. CTR; 3.98% in BP vs. CTR). Moreover, no probes from each of the three tested models reached significance level post FDR correction. However, when following a validation approach, one of the highest ranked probes, cg15021292, harbouring *PIK3R1*, was reported to show significantly differential methylation in major psychosis patients.

To our knowledge, our study is the first to deeply profile the cerebellar methylome in SZ and BP using the EPIC array. The inconsistencies between these two studies could be due to the different coverage, as the 27k array includes only about 5% of the CpGs covered by the EPIC array. Yet another reason behind the lack of replication could be the statistical pipeline used in either study (preprocessing, filtering and quality control), and more importantly the covariates used in the regression models. To this end, correcting for cellular composition of the tissue is nowadays considered a default covariate. And while to date there are algorithms correcting for cellular heterogeneity [64], when applied to cerebellar tissue, they underperform. This could be explained by the fact that the cerebellum, and especially its cortex, lacks uniformity and its cytoarchitecture is highly heterogeneous [65]. Even if the two cohorts studied here were from the same

brain bank and possibly processed in a similar manner, it cannot be excluded that the samples provided are dissimilar concerning cellular composition. Here, in order to circumvent this caveat, we employed a surrogate variable analysis approach in order to capture this heterogeneity, making our analysis more optimal than those used previously.

Overall, we illustrate a list of methylomic “players”, different from the ones reported previously in cortical areas [2, 12, 21]. Such observation is to be expected as it has been previously noted that the cerebellum exhibits distinct methylomic profiles in comparison to cortical and subcortical structures, but also blood [66]. Therefore, we support that this study is invaluable as it allows deeper insight in the involvement of epigenetic mechanisms within the cerebellum in neuropsychiatric disorders. Some limitations of the study concern the phenotyping of the samples. Recently, the idea that SZ constitutes a single categorical disorder has faded and, inspired by van Os and others, SZ is now considered a syndrome within the psychosis spectrum [3]. Similarly, for BP, two differential phenotypes have been described, namely bipolar I with episodes of mania and bipolar II, with episodes of hypomania. Hence, narrowing down the psychosis spectrum to a single diagnosis, as for SZ and BP in the present study, conceals all the various endophenotypes that might be a result of diverse and distinct pathological/molecular mechanisms. In order to bypass such matter, additional clinical information would be crucial for the most accurate interrogation of each psychiatric phenotype/profile.

In conclusion, this study represents the first ever conducted EWAS on the cerebellum of patients with major psychosis, using the EPIC array, and further advocates the epigenetic theory of major psychosis by identifying genetic loci with aberrant methylation patterns in the cerebellum of BP and SZ in comparison to neurologically healthy controls. One of these loci, *ZFP57*, was replicated in a second, independent cohort. Such outcome suggests that this gene might contribute to the pathogenesis of major psychosis and could be an interesting candidate for further investigation especially in BP-related research lines. Hence, our study urges research not only on the functional repercussions of differential *ZFP57* methylation in psychiatric deviant disorders, but also on the role of the cerebellum in the development and course of these disorders.

	Control	Bipolar Disorder	Schizophrenia
	Discovery Cohort/ Array Collection		
N	31	31	29
M/F	24/7	15/16	21/8
Age	44.96 ±	46.16 ±	42.17 ±
<i>Mean ± SD</i>	7.62	10.72	8.93
PMI	29.94 ±	35.45 ±	31.24 ±
<i>Mean ± SD</i>	13.16	17.59	12.73
	Replication Cohort/ Neuropathology Collection		
N	15	15	15
M/F	6/9	6/9	6/9
Age	44.53 ±	42.33 ±	48.07 ±
<i>Mean ± SD</i>	13.11	11.72	10.66
PMI	32.53 ±	33.67 ±	23.73 ±
<i>Mean ± SD</i>	16.13	14.62	9.95

Table 1: Demographic information from the two independent cohorts used in this study. Cerebellum tissue from individuals diagnosed with bipolar disorder and schizophrenia, as well as controls was obtained from the Stanley Medical Research Institute. Displayed are the number of the samples per diagnosis, the distribution of sex, age and postmortem interval (PMI; hours).

Rank	ProbeID	Estimate	p value	q value	Chromosome	Gene Name	Probe Location
1	cg09481699	0.069	1.51E-06	0.54	17	CTD-2116F7.1	
3	cg04261247	-0.021	1.94E-06	0.54	18	LINC01443	Body
7	cg14088090	-0.034	1.09E-05	0.92	1	TMEM125	Body
9	cg01684722	-0.022	1.72E-05	0.92	6	TIAM2	5'UTR
10	cg04312970	-0.021	1.81E-05	0.92	6	RPS6KA2	
11	cg02458602	0.054	1.92E-05	0.92	15	RP11-509A17.3	
12	cg02641825	-0.030	1.96E-05	0.92	12	CRACR2A	Body
13	cg06371443	-0.024	2.06E-05	0.92	3	CASR	5'UTR
15	cg15620905	0.041	2.29E-05	0.92	1	PTPRF	
17	cg05293488	-0.030	2.45E-05	0.92	1	RP5-1198020.4	TSS1500
18	cg26432999	0.006	2.48E-05	0.92	5	SQSTM1	
19	cg18328206	0.014	2.72E-05	0.92	1	RASSF5	
20	cg01468514	0.037	2.75E-05	0.92	5	ANKRD33B	TSS1500
21	cg02033206	-0.042	2.77E-05	0.92	12	FOXP4	Body
22	cg14743553	-0.068	3.00E-05	0.92	15	MIR1268A	TSS1500
23	cg02078844	0.020	3.01E-05	0.92	5	SLC23A1	
24	cg15874926	0.004	3.36E-05	0.92	19	KXD1	
26	cg17459557	0.053	3.42E-05	0.92	6	NCOA7	
27	cg24045369	-0.007	3.71E-05	0.92	18	KCTD1	
29	cg21865845	-0.007	3.71E-05	0.92	3	ROB01	5'UTR
30	cg24542170	-0.049	3.74E-05	0.92	6	Xxbac-BPG308J9.3	5'UTR

Table 2: The thirty top-ranked major psychosis-associated methylated positions from the discovery cohort. Displayed for each probe are the regression estimate for the major psychosis-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 5'UTR, 5' untranslated regions; TSS, transcription start site

Rank	ProbeID	Estimate	p value	q value	Chromosome	Gene Name	Probe Location
1	cg01264936	-0.0316	1.00E-06	0.84	14	IGHV4-4	TSS1500
2	cg10081761	-0.0299	8.61E-06	1	11	ALDH3B2	TSS1500
4	cg25599673	0.0235	9.13E-06	1	3	ST6GAL1	
5	cg04292660	-0.0142	9.63E-06	1	4	LRAT	TSS1500
6	cg05326743	0.0089	1.31E-05	1	9	DAB2IP	Body
7	cg16226546	-0.0405	1.35E-05	1	15	OR4M2	
8	cg04006967	-0.0227	1.69E-05	1	4	LINC00955	Body
9	cg19275261	0.0023	1.73E-05	1	18	EPB41L3	TSS1500
10	cg08898859	0.0261	2.02E-05	1	9	SCAI	
12	cg08404345	0.0184	2.20E-05	1	12	METTL20	
13	cg00995798	-0.0033	2.36E-05	1	2	TTC27	TSS1500
14	cg03209103	0.0244	2.66E-05	1	7	SGCE	
15	cg15919165	-0.0197	3.15E-05	1	2	AC099344.3	TSS200
16	cg17437033	0.0041	3.21E-05	1	5	KIF3A	Body
17	cg04741688	0.0124	3.28E-05	1	9	AK8	Body
18	cg25849390	-0.0206	3.38E-05	1	7	CCT6A	
19	cg11206763	0.0026	3.43E-05	1	12	ESYT1	TSS1500
20	cg19724470	0.0383	3.56E-05	1	9	CD274	5'UTR
21	cg08477215	0.0030	3.83E-05	1	7	POM121C	5'UTR
22	cg15059176	0.0202	4.04E-05	1	1	PRDM16	
23	cg13371675	-0.0152	4.07E-05	1	5	TPPP	Body
26	cg17033087	-0.0238	4.50E-05	1	10	DMBT1	
27	cg15874926	0.0024	4.61E-05	1	19	KXD1	
29	cg09481699	0.0329	5.21E-05	1	17	CTD-2116F7.1	TSS1500
30	cg22499215	0.0136	5.24E-05	1	22	GNB1L	3'UTR

Table 3: The thirty top-ranked bipolar disorder-associated methylated positions from the discovery cohort. Displayed for each probe are the regression estimate for the bipolar disorder-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 3'UTR, 3' untranslated region; 5'UTR, 5' untranslated region; TSS, transcription start site

Rank	Probe ID	Estimate	p value	FDR p value	Chromosome	Gene Name	Probe Location
3	cg00031013	0.047	4.04E-06	0.42	17	KDM6B	3'UTR
4	cg03390636	0.024	4.41E-06	0.42	15	ARNT2	Body
5	cg06465080	-0.008	4.97E-06	0.42	17	TXNDC17	1stExon
6	cg04117986	0.030	8.98E-06	0.49	5	PDGFRB	5'UTR
7	cg09443484	0.067	1.06E-05	0.49	11	C11orf41	Body
8	cg20483962	-0.014	1.17E-05	0.49	17	DVL2	TSS1500
9	cg05855039	0.008	1.35E-05	0.49	14	FLRT2	1stExon
10	cg02771673	0.050	1.45E-05	0.49	13	PCCA	Body
11	cg24768463	0.050	1.60E-05	0.49	12	ATN1	Body
12	cg18463686	0.021	1.63E-05	0.49	7	CLEC5A	5'UTR
13	cg25616787	-0.027	1.80E-05	0.49	1	SPSB1	5'UTR
14	cg00159799	-0.034	1.82E-05	0.49	19	PKN1	Body
15	cg07216258	0.018	1.99E-05	0.49	3	NFKBIZ	5'UTR
16	cg24923700	-0.010	2.00E-05	0.49	3	PLS1	TSS200
18	cg08525922	0.062	2.10E-05	0.49	3	VGLL4	Body
19	cg22331165	-0.020	2.28E-05	0.50	17	PLXDC1	Body
20	cg05509800	-0.027	2.52E-05	0.50	12	ALKBH2	TSS1500
22	cg25214914	0.051	2.89E-05	0.50	1	RXFP4	TSS1500
23	cg20263435	0.005	2.94E-05	0.50	8	AGPAT6	TSS1500
24	cg18686270	0.041	3.22E-05	0.50	3	PLSCR1	5'UTR
25	cg18238808	0.026	3.40E-05	0.50	17	CDC42EP4	3'UTR
26	cg23687971	-0.044	3.48E-05	0.50	20	NECAB3	TSS1500
27	cg01783706	0.009	3.50E-05	0.50	12	DNAJC22	TSS1500
28	cg15756091	-0.031	3.53E-05	0.50	1	RGSL1	Body
29	cg21555346	0.030	3.55E-05	0.50	15	MYO5A	Body

Table 4: The thirty top-ranked schizophrenia-associated methylated positions from the discovery cohort. Displayed for each probe are the regression estimate for the schizophrenia-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 5'UTR, 5' untranslated region; TSS, transcription start site

Chromosome	Start	End	Nr Probes	p value	Šidák-p	Gene Name	Gene Feature	Change
Psychiatric Phenotype								
chr6	29648161	29648902	20	2.49E-14	2.81E-11	ZFP57	intergenic	↑
chr1	205819088	205819610	11	1.28E-12	2.05E-09	PM20D1		↑
chr12	81110496	81110894	9	8.56E-11	1.80E-07	MYF5	TSS+utr5+cds	↑
chr1	44087631	44087824	5	3.90E-08	0.00017	PTPRF	utr3+cds	↑
chr2	200329654	200329943	9	6.77E-08	0.00020	SATB2		↑
Disorder Diagnosis								
Bipolar Disorder								
chr1	205819088	205819610	11	1.28E-10	2.05E-07	PM20D1		↑
chr12	81110605	81110777	6	1.13E-07	0.000548	MYF5	TSS+exon+utr5	↑
chr6	29648345	29648605	12	8.71E-07	0.002797	ZFP57	intergenic	↑
Schizophrenia								
chr1	205819088	205819610	11	1.11E-14	1.78E-11	PM20D1		↑
chr6	29648161	29648902	20	4.63E-14	5.22E-11	ZFP57	intergenic	↑
chr6	33288296	33288600	5	9.46E-09	2.6E-05	DAXX	intron+cds	↓
chr13	24844684	24844939	7	1.08E-08	3.56E-05	SPATA13		↑
chr14	106938289	106938622	8	2.87E-08	7.21E-05	LINC00221	nc_exon	↑
chr8	2585945	2586226	5	5.71E-08	0.00017	LOC101927815	nc_exon	↑

Table 5: Identification of probes in differentially methylated regions associated with major psychosis, bipolar disorder and schizophrenia from the discovery cohort. Displayed for each probe are the chromosomal position (genome build 37), the number of probes in the region (Nr Probes), the p-value and multiple testing-corrected p (Šidák-p), UCSC gene name, gene feature, and directionality of modification changes with the progression of Braak stage (up or down). TSS, transcription start site; utr5, 5' untranslated region; cds, coding sequence.

Rank	ProbeID	Estimate	p value	q value	Chromosome	Gene Name	Probe Location
1	cg20483962	-0.013	1.26E-07	0.041	17	DVL2	TSS1500
2	cg01221018	-0.038	1.93E-07	0.041	14	MDP	Body
6	cg01463828	-0.066	4.01E-06	0.269	8	PDLIM2	Body
7	cg26168557	-0.005	4.47E-06	0.269	13	SUGT1	TSS200
9	cg15756091	-0.027	6.05E-06	0.277	1	RGSL1	Body
10	cg22184944	0.057	6.59E-06	0.277	17	BAIAP2	Body
11	cg25192081	0.019	1.02E-05	0.343	1	JAK1	Body
12	cg02291619	0.024	1.11E-05	0.343	5	ARHGAP26	Body
13	cg04337828	0.073	1.14E-05	0.343	16	BANP	5'UTR
15	cg12634591	0.014	1.41E-05	0.395	2	HOXD3	TSS200
16	cg04392082	-0.030	1.78E-05	0.444	4	SORBS2	5'UTR
17	cg17456675	0.039	2.05E-05	0.444	1	MIB2	Body
18	cg20952901	0.055	2.14E-05	0.444	19	ABHD8	Body
19	cg12840245	0.033	2.31E-05	0.444	4	PCDH7	Body
20	cg12733079	0.011	2.35E-05	0.444	7	DOCK4	Body
21	cg16677448	0.060	2.40E-05	0.444	19	DMKN	TSS200
22	cg21666889	-0.011	2.41E-05	0.444	3	TMEM111	TSS1500
23	cg22185879	-0.027	2.60E-05	0.444	20	PPDPF	Body
24	cg06465080	-0.006	2.75E-05	0.444	17	TXNDC17	1stExon
25	cg03679544	0.037	2.81E-05	0.444	6	TIAM2	Body
26	cg06203102	-0.028	2.91E-05	0.444	1	TRIM46	Body
27	cg08525922	0.051	3.07E-05	0.444	3	VGLL4	Body
28	cg26702929	-0.009	3.16E-05	0.444	8	HR	1stExon
29	cg08396863	0.023	3.50E-05	0.444	2	TUBA4B	TSS1500
30	cg10327320	-0.013	3.56E-05	0.444	1	FAM54B	TSS200

Table 6: The thirty top-ranked major psychosis-associated methylated positions from the replication cohort. Displayed for each probe are the regression estimate for the major psychosis-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 5'UTR, 5' untranslated regions; TSS, transcription start site

Rank	Probe ID	Estimate	p value	q value	Chromosome	Gene Name	Probe Location
1	cg21813112	0.022	2.02E-06	0.45	1	TRIM33	TSS1500
2	cg25675800	0.033	4.03E-06	0.45	19	RINL	Body
3	cg12875426	0.034	4.55E-06	0.45	18	DYM	TSS1500
7	cg21609584	-0.018	8.72E-06	0.45	2	NPAS2	5'UTR
8	cg15158859	-0.047	1.01E-05	0.45	10	JAKMIP3	Body
9	cg01691987	0.015	1.01E-05	0.45	17	CHD3	Body
10	cg19682962	-0.008	1.06E-05	0.45	22	DRG1	Body
11	cg07271869	0.012	1.39E-05	0.51	3	PCYT1A	Body
12	cg03625807	-0.010	1.69E-05	0.51	16	GTF3C1	Body
13	cg04094640	-0.018	1.74E-05	0.51	16	NSMCE1	Body
14	cg13968557	0.022	1.79E-05	0.51	11	AP2A2	Body
15	cg13810457	-0.017	1.98E-05	0.51	14	TC2N	5'UTR
16	cg14825413	-0.073	2.08E-05	0.51	8	GLI4	Body
18	cg02495569	0.017	2.20E-05	0.51	5	NEURL1B	Body
19	cg01192748	0.021	2.87E-05	0.63	3	MCF2L2	Body
20	cg15155559	-0.025	3.70E-05	0.78	3	ULK4	Body
23	cg07963363	0.017	4.97E-05	0.91	8	RUNX1T1	Body
25	cg25363336	-0.019	6.04E-05	0.97	5	MYO10	Body
26	cg02722672	-0.025	6.40E-05	0.97	4	GRK4	Body
29	cg18577128	-0.009	6.89E-05	0.97	1	MAST2	TSS200
30	cg17420218	-0.006	7.44E-05	0.97	2	TTC32	TSS1500

Table 7: The thirty top-ranked bipolar disorder-associated methylated positions from the replication cohort. Displayed for each probe are the regression estimate for the bipolar disorder-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 5'UTR, 5' untranslated region; TSS, transcription start site

Rank	ProbeID	Estimate	p value	Q value	Chromosome	Gene Name	Probe Location
3	cg00031013	0.047	4.04E-06	0.42	17	KDM6B	3'UTR
4	cg03390636	0.024	4.41E-06	0.42	15	ARNT2	Body
5	cg06465080	-0.008	4.97E-06	0.42	17	TXNDC17	1stExon
6	cg04117986	0.030	8.98E-06	0.49	5	PDGFRB	5'UTR
7	cg09443484	0.067	1.06E-05	0.49	11	C11orf41	Body
8	cg20483962	-0.014	1.17E-05	0.49	17	DVL2	TSS1500
9	cg05855039	0.008	1.35E-05	0.49	14	FLRT2	1stExon
10	cg02771673	0.050	1.45E-05	0.49	13	PCCA	Body
11	cg24768463	0.050	1.60E-05	0.49	12	ATN1	Body
12	cg18463686	0.021	1.63E-05	0.49	7	CLEC5A	5'UTR
13	cg25616787	-0.027	1.80E-05	0.49	1	SPSB1	5'UTR
14	cg00159799	-0.034	1.82E-05	0.49	19	PKN1	Body
15	cg07216258	0.018	1.99E-05	0.49	3	NFKBIZ	5'UTR
16	cg24923700	-0.010	2.00E-05	0.49	3	PLS1	TSS200
18	cg08525922	0.062	2.10E-05	0.49	3	VGLL4	Body
19	cg22331165	-0.020	2.28E-05	0.50	17	PLXDC1	Body
20	cg05509800	-0.027	2.52E-05	0.50	12	ALKBH2	TSS1500
22	cg25214914	0.051	2.89E-05	0.50	1	RFXFP4	TSS1500
23	cg20263435	0.005	2.94E-05	0.50	8	AGPAT6	TSS1500
24	cg18686270	0.041	3.22E-05	0.50	3	PLSCR1	5'UTR
25	cg18238808	0.026	3.40E-05	0.50	17	CDC42EP4	3'UTR
26	cg23687971	-0.044	3.48E-05	0.50	20	NECAB3	TSS1500
27	cg01783706	0.009	3.50E-05	0.50	12	DNAJC22	TSS1500
28	cg15756091	-0.031	3.53E-05	0.50	1	RGS1	Body
29	cg21555346	0.030	3.55E-05	0.50	15	MYO5A	Body

Table 8: The thirty top-ranked schizophrenia-associated methylated positions from the replication cohort. Displayed for each probe are the regression estimate for the bipolar disorder-associated analysis (Estimate), the corresponding p value, the FDR-corrected p value (q value), the residing chromosome as well as the Illumina gene annotation (Gene Name) and the location of the probe within the genetic locus. 3'UTR, 3' untranslated region; 5'UTR, 5' untranslated region; TSS, transcription start site

Chromosome	Start	End	Nr Probes	p value	Šidák-p	Gene Name	Gene Feature	Change
Psychiatric Phenotype								
chr19	36004233	36004995	5	7.28E-10	4.01E-07	DMKN	TSS+utr5+cds	↑
chr11	1327962	1328487	4	2.62E-09	2.10E-06	TOLLIP	intron	↑
chr8	21608193	21608281	3	1.69E-09	8.06E-06	GFRA2	cds	↓
chr17	79031091	79031713	7	4.30E-08	2.90E-05	BAIAP2	intron+cds	↑
chr5	177853481	177853643	3	1.24E-08	3.22E-05	COL23A1	intron	↓
Disorder Diagnosis								
Bipolar Disorder								
chr6	29648161	29648757	18	3.78E-26	2.66E-23	ZFP57	intergenic	↑
chr12	54446019	54446309	7	1.51E-08	2.19E-05	HOXC4	intron+utr5	↑
Schizophrenia								
chr12	58013636	58013916	6	1.95E-12	2.93E-09	SLC26A10	TSS+exon+utr5	↑
chr11	5706226	5706436	7	1.55E-09	3.11E-06	TRIM5	TSS+exon+utr5	↑
chr4	7762596	7762706	3	3.17E-09	1.21E-05	AFAP1	nc_intron+exon+utr3	↑
chr1	55271673	55271928	5	1.07E-08	1.75E-05	LEXM	TSS+intron+utr5+cds	↓
chr17	37123638	37123950	9	2.94E-08	3.95E-05	FBXO47	TSS+exon+utr5	↓
chr6	164241327	164241414	3	3.11E-08	0.00015	LOC102724152	intergenic	↑
chr1	228657536	228657763	3	9.79E-08	0.000181	MIR4666A	intergenic	↓
chr8	142368026	142368333	6	1.35E-07	0.000185	GPR20	intron+exon+utr5	↓
chr6	161860263	161860562	3	2.46E-07	0.000346	PRKN	intron	↓
chr2	239008943	239009247	6	7.67E-07	0.001059	ESPNL	TSS+utr5+cds	↓
chr2	38893161	38893252	3	3.66E-07	0.00169	GALM	exon+utr5	↓
chr8	144779248	144779377	3	1.03E-06	0.003358	BREA2	nc_exon	↑

Table 9: Identification of probes in differentially methylated regions associated with major psychosis, bipolar disorder and schizophrenia from the replication cohort. Displayed for each probe are the chromosomal position (genome build 37), the number of probes in the region (Nr Probes), the p-value and multiple testing-corrected p (Šidák-p), UCSC gene name, gene feature, and directionality of modification changes with the each relevant psychiatric diagnosis. TSS, transcription start site; utr5, 5' untranslated region; cds, coding sequence.

Supplementary Figure 1: Density plots of normalized beta values for methylated cytosines from the discovery (A) and replication (B) cohort. Plots include all probes used for the analysis.

Figure 1: Manhattan and QQ plots of the global methylation regression analyses from the discovery cohort. In the Manhattan plots (A-C) the blue line indicates the nominal significance threshold of 0.05 and the red one illustrates the genome-wide significance threshold. For the QQ plots (a-c) the fit of each model was evaluated by the genomic inflation factor λ , which is depicted on the right side of each plot. (A,a) Psychiatric Phenotype, (B,b) Bipolar Disorder, (C,c) Schizophrenia

A

a

B

b

C

c

Figure 2: Manhattan and QQ plots of the global methylation regression analyses from the replication. In the Manhattan plots (A-C) the blue line indicates the nominal significance threshold of 0.05 and the red one illustrates the genome-wide significance threshold. For the QQ plots (a-c) the fit of each model was evaluated by the genomic inflation factor λ , which is depicted on the right side of each plot. (A,a) Psychiatric Phenotype, (B,b) Bipolar Disorder, (C,c) Schizophrenia

Figure 3: Methylation changes with the diagnosis of major psychosis of the two top-ranked probes (cg20483962 [A], cg1221018 [B]) annotating for DVL2 and MDP, respectively. The y – axis represents beta values regressed for age, sex, batch effect and 5 surrogate variables.

Figure 4: Schizophrenia-associated DNA methylation estimates for the top-ranked DMPs shared between the Array discovery cohort and the Neuropathology replication cohort are significantly correlated ($r = 0.26$, $p = 0.046$). SZ, schizophrenia

ID	Description	Ontology	Genes in term	Genes altered	Gene names	P	FDR
GO:0010842	retina layer formation	BP	21	6	FOXN4; DSCAM; SDK1; PTF1A; TFAP2A; TFAP2B	5.8E-04	1
GO:0018279	protein N-linked glycosylation via asparagine	BP	39	6	ST3GAL6; ST6GAL1; ST3GAL1; ST3GAL3; UBE2G2; MCFD2	2.4E-03	1
GO:0051451	myoblast migration	BP	10	3	PLEKH01; SIX4; ITGB1BP1	2.8E-03	1
GO:0006054	N-acetylneuraminate metabolic process	BP	10	3	AMDH02; ST6GAL1; ST3GAL1	3.3E-03	1
GO:0003407	neural retina development	BP	52	7	FOXN4; DSCAM; SDK1; PTF1A; GPM6A; TFAP2A; TFAP2B	4.1E-03	1
GO:0097581	lamellipodium organization	BP	68	9	VAV3; DNMT2; SPATA13; FER; KANK1; PLEKH01; LRRC16A; CDDC88A; HDAC4	5.9E-03	1
GO:0032680	regulation of tumor necrosis factor production	BP	98	8	CHRNA7; CLU; ZBTB20; FOXP1; NFKB1L1; GHR1; AXL; CCL2	7.1E-03	1
GO:0046676	negative regulation of insulin secretion	BP	36	5	BMP8A; GHR1; PPP3CA; ENY2; SFRP1	7.5E-03	1
GO:0032640	tumor necrosis factor production	BP	100	8	CHRNA7; CLU; ZBTB20; FOXP1; NFKB1L1; GHR1; AXL; CCL2	7.7E-03	1
GO:0060042	retina morphogenesis in camera-type eye	BP	45	6	FOXN4; DSCAM; SDK1; PTF1A; TFAP2A; TFAP2B	8.4E-03	1
GO:0005794	Golgi apparatus	CC	1374	69	AKT3; CHST4; TMEM5; ST3GAL6; SYNRG; OSBP19; CLCN3; CLU; CLTC; COL1A1; AP1S3; SELM; CSPG4; MANEAL; AP1B1; DNMT2; ABCA1; SLC39A11; MECOM; EXT1; FASN; SEC31A; PDXD1; GGA2; OSL1; GSGT1; GOLIM4; PACSIN1; ICA1; TMEM173; GALNT4; AGRN; ARF6; MYO18A; NOTCH4; SOST; SDF4; RAB6B; PI4KA; PKD1; PLAGL1; IL17RD; PACS1; CDDC88A; PRKCE; PRKG1; MAN1C1; TBC1D14; XYLT1; ST6GAL1; ST3GAL1; ST3GAL3; SLC1A6; CHID1; SYT1; TFAP2A; WNT3; C6orf25; YIPF5; TRAPPC9; CALN1; NCALD; EPCAB48; SGCE; MCFD2; VAMP3; CABP1; ENTPD6; SEC16A	4.6E-03	1
GO:0031300	intrinsic component of organelle membrane	CC	151	12	CHST4; ST3GAL6; P2RX2; ATP13A2; ATP6V1G2; GDAP1; MAN1C1; ST6GAL1; ST3GAL1; ST3GAL3; PEX11B; MCOU	4.8E-03	1
GO:0030130	clathrin coat of trans-Golgi network vesicle	CC	12	3	SYNRG; CLTC; NCALD	8.0E-03	1
GO:0030139	endocytic vesicle	CC	240	17	CLCN3; CLTC; AP1S3; DAB2IP; ATP6V0E2; AP1B1; DLG4; DNMT2; ABCA1; GOLIM4; ARF6; LRP1; CCL2; RAB17; WNT3; AMN; MTSS1	8.6E-03	1
GO:0031301	integral component of organelle membrane	CC	145	11	ST3GAL6; P2RX2; ATP13A2; ATP6V1G2; GDAP1; MAN1C1; ST6GAL1; ST3GAL1; ST3GAL3; PEX11B; MCOU	9.8E-03	1
GO:0012510	trans-Golgi network transport vesicle membrane	CC	14	3	SYNRG; CLTC; NCALD	9.9E-03	1
GO:0005798	Golgi-associated vesicle	CC	78	7	SYNRG; CLTC; PACSIN1; PI4KA; PKD1; PACS1; NCALD; CHST4; STAM2; TMEM5; TRDN; ST3GAL6; SYNRG; OSBP19; CLCN3; CLTC; AP1S3; MANEAL; ATP6V0E2; AP1B1; PKD1L1; DLG4; DNMT2; EXT1; MARCH8; SEC31A; GGA2; SARMT1; ATP11A; ATP13A2; GSGT1; GOLIM4; GNAT1; GNBL1; EHDA; ACACB; ICA1; TMEM173; GALNT4; AGRN; MYO18A; LRP1; NOTCH4; RAB6B; PDE8B; ATP5G3; PI4KA; PKD1; ATP6V1G2; GDAP1; IL17RD; SLC29A3; KIF18B; MAN1C1; RPTOR; RYR3; XYLT1; RAB17; ST6GAL1; ST3GAL1; ST3GAL3; SRP9; SRPR; SYN2; SYT1; WNT3; C19orf50; KREMEN2; YIPF5; AMN; SBF2; TRAPPC9; CALN1; NCALD; PEX11B; ATG16L2; MCFD2; VAMP3; CABP1; ENTPD6; PHACTR2; SEC16A	0.012	1
GO:0044431	Golgi apparatus part	CC	855	44	CHST4; TMEM5; ST3GAL6; SYNRG; CLCN3; CLTC; AP1S3; CSPG4; MANEAL; AP1B1; DNMT2; EXT1; SEC31A; GGA2; GSGT1; GOLIM4; PACSIN1; ICA1; GALNT4; AGRN; MYO18A; NOTCH4; SDF4; RAB6B; PI4KA; PKD1; IL17RD; PACS1; MAN1C1; XYLT1; ST6GAL1; ST3GAL1; ST3GAL3; CHID1; WNT3; YIPF5; TRAPPC9; CALN1; NCALD; MCFD2; VAMP3; CABP1; ENTPD6; SEC16A	0.014	1
GO:0030660	Golgi-associated vesicle membrane	CC	49	5	SYNRG; CLTC; PI4KA; PKD1; NCALD	0.016	1
GO:0061650	ubiquitin-like protein conjugating enzyme activity	MF	27	7	UBE2C; UBE2J2; UBE2QL1; UBE20; UBE2S1; UBE2G2; UBE2J	0.000	0.295
GO:0070402	NADPH binding	MF	13	3	FASN; MTRR; SRD5A1	0.007	1
GO:0022892	substrate-specific transporter activity	MF	1063	52	SLC25A13; SLC46A1; CHRNA7; CLCN3; SLC36A4; AP1S3; TMEM37; ATP6V0E2; AP1B1; PKD1L1; DLG4; ABCA1; SLC39A11; RASA3; P2RX2; MLC1; ATP11A; ATP13A2; NNT; PITPN1; GPM6A; GRIK4; GRIK5; TRPM5; SLC26A5; KCNQ1; ATP2B2; SLC22A18; IPO11; ATP5G3; PKD1; ATP6V1G2; SLC29A3; ANKH; SLC7A10; PLSCR2; ANO2; NIPAL3; RAB4A; RYR3; SCN4B; SLC1A6; CACNA1S; NCALD; ABCO11; CACNA1G; MCOU; SLC6A5; RFT1; ITGB1BP1; CACNA2D4; SLC23A1	0.008	1
GO:0008373	sialyltransferase activity	MF	21	4	ST3GAL6; ST6GAL1; ST3GAL1; ST3GAL3	0.016	1
GO:0005215	transporter activity	MF	1238	56	SLC25A13; CPLX1; SLC46A1; CHRNA7; CLCN3; SLC36A4; AP1S3; TMEM37; ATP6V0E2; AP1B1; PKD1L1; DLG4; ABCA1; SLC39A11; RASA3; P2RX2; MLC1; ATP11A; ATP13A2; NNT; PITPN1; SEC22A; GPM6A; GRIK4; GRIK5; TRPM5; SLC26A5; KCNQ1; LRP1; ATP2B2; SLC22A18; IPO11; ATP5G3; PKD1; ATP6V1G2; SLC29A3; ANKH; SLC7A10; PLSCR2; ANO2; NIPAL3; RAB4A; RYR3; SCN4B; STRA6; SLC1A6; CACNA1S; NCALD; ABCO11; CACNA1G; MCOU; SLC6A5; RFT1; ITGB1BP1; CACNA2D4; SLC23A1	0.017	1
GO:0015075	ion transmembrane transporter activity	MF	762	39	SLC25A13; SLC46A1; CHRNA7; CLCN3; TMEM37; ATP6V0E2; PKD1L1; DLG4; ABCA1; SLC39A11; RASA3; P2RX2; ATP13A2; NNT; GPM6A; GRIK4; GRIK5; TRPM5; SLC26A5; KCNQ1; ATP2B2; SLC22A18; ATP5G3; PKD1; ATP6V1G2; ANKH; ANO2; NIPAL3; RYR3; SCN4B; SLC1A6; CACNA1S; NCALD; ABCO11; CACNA1G; MCOU; SLC6A5; CACNA2D4; SLC23A1	0.020	1
GO:0072341	modified amino acid binding	MF	62	6	SLC46A1; OSBP19; FASN; GNM1; AXL; SYT1	0.021	1
GO:0016741	transferase activity, transferring one-carbon groups	MF	214	13	PRMT3; PRDM14; MECOM; EZH1; METTL20; GNM1; AMT; MLL; ATIC; NSUN2; PRDM13; PRDM16; THUMP2	0.022	1
GO:0005183	vitamin transporter activity	MF	23	3	SLC46A1; STRA6; SLC23A1	0.026	1
GO:0005542	folic acid binding	MF	11	2	SLC46A1; GNM1	0.027	1

Supplementary Table 2: Gene Ontology (GO) enrichment analysis results for the top 858 major psychosis-associated top-ranked differentially methylated positions. Displayed for each GO term is the ID, description, the ontology type, the number of genes in the term, the number of the genes altered from the input list as well as their names, p-value (p) and FDR corrected p value (FDR). BP, biological process; CC, cellular component; MF, molecular function

ID	Description	Ontology	Genes in term	Genes altered	Gene names	P	FDR
GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	BP	151	19	SDK1; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; ROBO1; PCDHGB4; CELSR1; PCDHGA8	2.70E-05	0.21
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	BP	216	21	SDK1; CLDN15; FXYD5; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; ROBO1; PCDHGB4; CELSR1; PCDHGA8	1.25E-04	0.49
GO:0031954	positive regulation of protein autophosphorylation	BP	22	5	TOM1L1; RAD50; PDGFA; PDGFB; PDGFC	6.62E-04	1
GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	BP	101	9	TIRAP; CLU; FOXP1; CD274; ISL1; AXL; TRIM27; CCL2; TNFRSF8	0.0013	1
GO:0043496	regulation of protein homodimerization activity	BP	19	4	TIRAP; ISL1; ITGA4; TRAF2	0.0020	1
GO:0045740	positive regulation of DNA replication	BP	53	6	IGF1R; PDGFA; PDGFB; PDGFC; TGFβ3; E2F8	0.0041	1
GO:0032680	regulation of tumor necrosis factor production	BP	98	8	TIRAP; CLU; FOXP1; ISL1; AXL; TRIM27; CCL2; TNFRSF8	0.0041	1
GO:006275	regulation of DNA replication	BP	110	9	WAPAL; HCR7; IGF1R; PDGFA; PDGFB; TIPIN; PDGFC; TGFβ3; E2F8	0.0042	1
GO:0032640	tumor necrosis factor production	BP	100	8	TIRAP; CLU; FOXP1; ISL1; AXL; TRIM27; CCL2; TNFRSF8	0.0045	1
GO:0051054	positive regulation of DNA metabolic process	BP	190	13	RAD50; RNF168; EYA2; FGFR4; TINF2; IGF1R; PDGFA; PDGFB; PRKQC; PDGFC; TGFβ3; E2F8; CCT6A	0.0046	1
GO:0090665	glycoprotein complex	CC	19	4	SGCA; SNTB1; UTRN; SGCE	0.0053	1
GO:0034774	secretory granule lumen	CC	81	6	CLU; IGFB2; PDGFA; PDGFB; RARRES2; TGFβ3	0.0106	1
GO:0031527	filopodium membrane	CC	16	3	SYNE2; ARF6; UTRN	0.0221	1
GO:0008540	proteasome regulatory particle, base subcomplex	CC	11	2	PSMD1; PSMD9	0.0222	1
GO:0060205	cytoplasmic membrane-bounded vesicle lumen	CC	100	6	CLU; IGFB2; PDGFA; PDGFB; RARRES2; TGFβ3	0.0228	1
GO:0030141	secretory granule	CC	334	15	CLCN3; CLU; DMBT1; UNC13D; FSTL4; HCR7; IGFB2; PDGFA; PDGFB; RARRES2; SYPL1; TGFβ3; SYT7; VAMP3; PHACTR2	0.0307	1
GO:0030139	endocytic vesicle	CC	240	14	TIRAP; CLCN3; CLTC; DAB2IP; DMBT1; GOLIM4; ARF6; LRP1; MYO6; RPS27A; CCL2; WNT3; WNT7A; SYT7	0.0310	1
GO:0005885	Arp2/3 protein complex	CC	11	2	ARPC18; ACTR38	0.0322	1
GO:0048188	Set1C/COMPASS complex	CC	10	2	DYDC1; DYDC2	0.0350	1
GO:0044232	organelle membrane contact site	CC	11	2	ESYT1; ESYT3	0.0438	1
GO:0016279	protein-lysine N-methyltransferase activity	MF	55	7	DYDC1; EEF2KMT; MECOM; EZH1; METTL20; PRDM16; DYDC2	0.0041	1
GO:0031996	thioesterase binding	MF	13	3	ARF6; TRAF2; TRAF3	0.0042	1
GO:0016278	lysine N-methyltransferase activity	MF	56	7	DYDC1; EEF2KMT; MECOM; EZH1; METTL20; PRDM16; DYDC2	0.0043	1
GO:0008170	N-methyltransferase activity	MF	85	8	DYDC1; EEF2KMT; MECOM; EZH1; METTL20; GNMT; PRDM16; DYDC2	0.0086	1
GO:0005509	calcium ion binding	MF	670	35	DLL3; FBLN7; DGKG; FSTL4; ZFEB1; PADI4; HRNR; LRP1; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; ODZ2; S100A4; SRR; SGCA; LRP8; EFCAB7; EFCAB4B; PCDHGB4; SYT7; NRXN1; CELSR1; PLCH2; PCDHGA8	0.0167	1
GO:0008276	protein methyltransferase activity	MF	81	7	DYDC1; EEF2KMT; MECOM; EZH1; METTL20; PRDM16; DYDC2	0.0208	1
GO:0061778	intracellular chloride channel activity	MF	16	3	ANO4; ANO1; TTYH3	0.0219	1
GO:0008757	S-adenosylmethionine-dependent methyltransferase activity	MF	139	9	DYDC1; WBSR27; EEF2KMT; MECOM; EZH1; METTL20; GNMT; PRDM16; DYDC2	0.0250	1
GO:0004714	transmembrane receptor protein tyrosine kinase activity	MF	64	7	EPHB1; FGFR4; IGF1R; LTK; ROR1; AXL; TRIM27	0.0288	1
GO:0018024	histone-lysine N-methyltransferase activity	MF	44	5	DYDC1; MECOM; EZH1; PRDM16; DYDC2	0.0299	1

Supplementary Table 3: Gene Ontology (GO) enrichment analysis results for the top 753 bipolar disorder-associated top-ranked differentially methylated positions. Displayed for each GO term is the ID, description, the ontology type, the number of genes in the term, the number of the genes altered from the input list as well as their names, p-value (p) and FDR corrected p value (FDR). BP, biological process; CC, cellular component; MF, molecular function

ID	Description	Ontology	Genes in term	Genes altered	Gene names	P	FDR
GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	BP	151	27	CDH4; SDK1; CADM2; PCDH7; PKD1; DCHS2; PCDHGB7; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; DSCAML1; ROBO1; FAT4; KIRREL3; PCDHGB4; PCDHGA8	2.27E-08	0.0002
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	BP	216	30	CDH4; ODZ3; DAB1; SDK1; LPHN1; CADM2; PCDH7; PKD1; DCHS2; PCDHGB7; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; DSCAML1; ROBO1; FAT4; KIRREL3; PCDHGB4; PCDHGA8	1.84E-07	0.0007
GO:0072170	metanephric tubule development	BP	23	6	WWTR1; PKD1; LGR4; WNT9B; PAX8; FAT4	2.95E-04	0.58
GO:0072173	metanephric tubule morphogenesis	BP	11	4	PKD1; LGR4; WNT9B; PAX8	0.001	1
GO:0072234	metanephric nephron tubule development	BP	20	5	WWTR1; PKD1; LGR4; PAX8; FAT4	0.001	1
GO:0071900	regulation of protein serine/threonine kinase activity	BP	445	32	ODZ3; CDK7; AKAP13; ADORA1; CSPG4; DAB2IP; DAXX; EFNA1; TAB2; GPS2; GSTP1; ITGA1; MNAT1; PRKAG2; GHR1; PKD1; PKIB; PRKAR1A; PRKAR1B; MAPK10; PROX1; MAP3K7CL; PTEN; RPTOR; WNK1; SORL1; MAP3K7; SESN2; PPP1R1B; TRAF7; CDK5R1; DIRAS3	0.002	1
GO:2000479	regulation of cAMP-dependent protein kinase activity	BP	14	4	PKIB; PRKAR1A; PRKAR1B; SESN2	0.003	1
GO:0072202	cell differentiation involved in metanephros development	BP	25	5	WWTR1; LGR4; WNT9B; PAX8; FAT4	0.004	1
GO:0050795	regulation of behavior	BP	60	7	ADORA1; DLG4; LEPR; ZFX3; GHR1; NR2C2; NRXN1	0.004	1
GO:0045835	negative regulation of meiotic nuclear division	BP	12	3	DMRT1; PRKAR1A; RPS6KA2	0.005	1
GO:0031588	nucleotide-activated protein kinase complex	CC	10	4	PRKAG2; PRKAB2; PRKAR1A; SESN2	0.000	0.58
GO:0005675	holo TFIIF complex	CC	10	3	CDK7; MNAT1; MMS19	0.005	1
GO:0016605	PML body	CC	91	10	PARK7; DAXX; ZNF451; NSMCE2; EIF3E; ARNTL; THAP1; PTEN; ODZ2; SQSTM1	0.008	1
GO:1902911	protein kinase complex	CC	84	9	CDK7; MNAT1; PRKAG2; PRKAB2; PRKAR1A; PRKAR1B; MMS19; MAP3K7; SESN2	0.013	1
GO:0005747	mitochondrial respiratory chain complex I	CC	41	4	PARK7; NDUFB2; NDUFS1; FOXRED1	0.028	1
GO:0032391	photoreceptor connecting cilium	CC	31	4	NPHP4; GNAT1; MYO7A; IFT140	0.033	1
GO:0097546	ciliary base	CC	26	4	NPHP4; GLI2; PRKAR1A; PRKAR1B	0.033	1
GO:0032806	carboxy-terminal domain protein kinase complex	CC	20	3	CDK7; MNAT1; MMS19	0.036	1
GO:1902554	serine/threonine protein kinase complex	CC	72	7	CDK7; MNAT1; PRKAR1A; PRKAR1B; MMS19; MAP3K7; SESN2	0.039	1
GO:0005793	endoplasmic reticulum-Golgi intermediate compartment	CC	103	8	RAB37; MYO18A; NUCB1; TMED7; RAB6B; ERGIC1; SURF4; MCFD2	0.040	1
GO:0030291	protein serine/threonine kinase inhibitor activity	MF	29	6	PRKAG2; PKIB; PRKAR1A; PRKAR1B; WNK1; PPP1R1B	0.001	1
GO:0016209	antioxidant activity	MF	75	8	TXNRD2; PARK7; GSTP1; GSTZ1; MPO; NXN; PXDN; SESN2	0.002	1
GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	MF	44	6	PARK7; GSTP1; GSTZ1; MPO; PXDN; SESN2	0.002	1
GO:0050681	androgen receptor binding	MF	39	6	CDK7; PARK7; DAXX; FOXF1; PRMT2; KDM3A; DAXX; EFNA5; PRKAG2; GHR1; RPTOR; WNK1; CDK5R1	0.008	1
GO:0030295	protein kinase activator activity	MF	56	7	CDK5R1	0.010	1
GO:0005509	calcium ion binding	MF	670	42	CDH4; CAPN11; DLL1; LRP1; LTBP1; ASPH; MYL2; NOTCH4; NUCB1; SPOCK3; PCDH7; DCHS2; PCDHGB7; PCDHGB6; PCDHGB5; PCDHGB3; PCDHGB2; PCDHGB1; PCDHGA10; PCDHGA9; PCDHGA7; PCDHGA6; PCDHGA5; PCDHGA4; PCDHGA3; PCDHGA2; PCDHGA1; ODZ2; RYR1; SLIT3; SPARC; C1S; FAT4; EFCAB4B; SYTL1; PCDHGB4; ACTN2; CDK5R1; CPNE1; MCFD2; NRXN1; PCDHGA8	0.014	1
GO:0008191	metalloendopeptidase inhibitor activity	MF	14	3	SPOCK3; TIMP2; RECK	0.015	1
GO:0004438	phosphatidylinositol-3-phosphatase activity	MF	11	3	PTEN; MTMR3; MTMR7	0.015	1
GO:0052743	inositol tetrakisphosphate phosphatase activity	MF	10	3	INPP5A; ITPK1; PTEN	0.016	1
GO:0019209	kinase activator activity	MF	63	7	DAXX; EFNA5; PRKAG2; GHR1; RPTOR; WNK1; CDK5R1	0.017	1

Supplementary Table 4: Gene Ontology (GO) enrichment analysis results for the top 897 major psychosis-associated top-ranked differentially methylated positions. Displayed for each GO term is the ID, description, the ontology type, the number of genes in the term, the number of the genes altered from the input list as well as their names, p-value (p) and FDR corrected p value (FDR). BP, biological process; CC, cellular component; MF, molecular function

References

1. Craddock, N., M.C. O'donovan, and M.J. Owen, Genes for schizophrenia and bipolar disorder? Implications for psychiatric nosology. *Schizophrenia bulletin*, 2006. 32(1): p. 9-16.
2. Mill, J., et al., Epigenomic Profiling Reveals DNA-Methylation Changes Associated with Major Psychosis. *American Journal of Human Genetics*, 2008. 82(3): p. 696-711.
3. Os, J.v., "Schizophrenia" does not exist. *BMJ*, 2016. 352.
4. McKusick, V.A., *Mendelian Inheritance in Man. A Catalog of Human Genes and Genetic Disorders*. Baltimore: Johns Hopkins University Press, 1998. 12.
5. Nöthen, M.M., et al., New findings in the genetics of major psychoses. *Dialogues in Clinical Neuroscience*, 2010. 12(1): p. 85-93.
6. Rodriguez-Murillo, L., J.A. Gogos, and M. Karayiorgou, The genetic architecture of schizophrenia: new mutations and emerging paradigms. *Annual review of medicine*, 2012. 63: p. 63-80.
7. Cardno, A.G. and Gottesman, II, Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. *Am J Med Genet*, 2000. 97(1): p. 12-7.
8. Craddock, N. and I. Jones, Genetics of bipolar disorder. *Journal of Medical Genetics*, 1999. 36(8): p. 585-594.
9. Dempster, E.L., et al., Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder. *Human molecular genetics*, 2011. 20(24): p. 4786-4796.
10. Montano, C., et al., Association of dna methylation differences with schizophrenia in an epigenome-wide association study. *JAMA Psychiatry*, 2016. 73(5): p. 506-514.
11. Aberg, K.A., et al., Methylome-wide association study of schizophrenia: Identifying blood biomarker signatures of environmental insults. *JAMA Psychiatry*, 2014. 71(3): p. 255-264.
12. Xiao, Y., et al., The DNA Methylome and Transcriptome of Different Brain Regions in Schizophrenia and Bipolar Disorder. *PLOS ONE*, 2014. 9(4): p. e95875.

13. Viana, J., et al., Schizophrenia-associated methylomic variation: molecular signatures of disease and polygenic risk burden across multiple brain regions. *Human molecular genetics*, 2016. 26(1): p. 210-225.
14. Chen, C., et al., Correlation between DNA methylation and gene expression in the brains of patients with bipolar disorder and schizophrenia. *Bipolar disorders*, 2014. 16(8): p. 790-799.
15. Phillips, J.R., et al., The Cerebellum and Psychiatric Disorders. *Frontiers in Public Health*, 2015. 3: p. 66.
16. Baldacara, L., et al., Is cerebellar volume related to bipolar disorder? *J Affect Disord*, 2011. 135(1-3): p. 305-9.
17. Smith, K.A., et al., Cerebellar responses during anticipation of noxious stimuli in subjects recovered from depression. *The British Journal of Psychiatry*, 2002. 181(5): p. 411-415.
18. Crespo-Facorro, B., et al., Neuropsychological functioning and brain structure in schizophrenia. *International Review of Psychiatry*, 2007. 19(4): p. 325-336.
19. Crespo-Facorro, B., et al., Neural basis of novel and well-learned recognition memory in schizophrenia: a positron emission tomography study. *Human brain mapping*, 2001. 12(4): p. 219-231.
20. Liang, M.J., et al., Identify changes of brain regional homogeneity in bipolar disorder and unipolar depression using resting-state fMRI. *PLoS One*, 2013. 8(12): p. e79999.
21. Chen, Y.L., et al., Resting-state fMRI mapping of cerebellar functional dysconnections involving multiple large-scale networks in patients with schizophrenia. *Schizophr Res*, 2013. 149(1-3): p. 26-34.
22. Turner, R. and A. Schiavetto, The cerebellum in schizophrenia: a case of intermittent ataxia and psychosis—clinical, cognitive, and neuroanatomical correlates. *The Journal of neuropsychiatry and clinical neurosciences*, 2004. 16(4): p. 400-408.
23. Yeganeh-Doost, P., et al., The role of the cerebellum in schizophrenia: from cognition to molecular pathways. *Clinics*, 2011. 66: p. 71-77.

24. Schmitt, A., et al., Gene expression of NMDA receptor subunits in the cerebellum of elderly patients with schizophrenia. *European Archives of Psychiatry and Clinical Neuroscience*, 2010. 260(2): p. 101-111.
25. Fatemi, S.H., et al., Expression of GABAA alpha2-, beta1- and epsilon-receptors are altered significantly in the lateral cerebellum of subjects with schizophrenia, major depression and bipolar disorder. *Transl Psychiatry*, 2013. 3: p. e303.
26. Fatemi, S.H., et al., GABAergic dysfunction in schizophrenia and mood disorders as reflected by decreased levels of glutamic acid decarboxylase 65 and 67 kDa and Reelin proteins in cerebellum. *Schizophrenia research*, 2005. 72(2): p. 109-122.
27. Smolin, B., et al., Differential expression of genes encoding neuronal ion-channel subunits in major depression, bipolar disorder and schizophrenia: implications for pathophysiology. *International Journal of Neuropsychopharmacology*, 2012. 15(7): p. 869-882.
28. Torrey, E.F., et al., The stanley foundation brain collection and neuropathology consortium. *Schizophr Res*, 2000. 44(2): p. 151-5.
29. Davis, S., et al., methylumi: Handle Illumina methylation data. R package version, 2014. 2(0).
30. Schalkwyk, L., et al., watermelon: Illumina 450 methylation array normalization and metrics. R package version, 2013. 1(2).
31. Blair, J.D. and E.M. Price, Illuminating potential technical artifacts of DNA-methylation array probes. *The American Journal of Human Genetics*, 2012. 91(4): p. 760-762.
32. Chen, Y.-a., et al., Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics*, 2013. 8(2): p. 203-209.
33. Turner, S.D., qqman: an R package for visualizing GWAS results using QQ and manhattan plots. *BioRxiv*, 2014: p. 005165.
34. Dabney, A., J.D. Storey, and G. Warnes, qvalue: Q-value estimation for false discovery rate control. R package version, 2010. 1(0).
35. Pedersen, B.S., et al., Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. *Bioinformatics*, 2012. 28(22): p. 2986-2988.

36. Phipson, B., J. Maksimovic, and A. Oshlack, *missMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform*. *Bioinformatics*, 2015. 32(2): p. 286-288.
37. Kirov, G., et al., *A genome-wide association study in 574 schizophrenia trios using DNA pooling*. *Molecular psychiatry*, 2009. 14(8): p. 796-803.
38. Ingason, A., et al., *Expression analysis in a rat psychosis model identifies novel candidate genes validated in a large case-control sample of schizophrenia*. *Translational Psychiatry*, 2015. 5(10): p. e656.
39. Sun, Y., et al., *The schizophrenia/bipolar disorder candidate gene GNB1L is regulated in human temporal cortex by a cis-acting element located within the 3'-region*. *Neuroscience Bulletin*, 2015. 31(1): p. 43-52.
40. Costas, J., et al., *Role of DISC1 interacting proteins in schizophrenia risk from genome-wide analysis of missense SNPs*. *Ann Hum Genet*, 2013. 77(6): p. 504-12.
41. Anjorin, A., et al., *DISC1 association, heterogeneity and interplay in schizophrenia and bipolar disorder*. *Molecular psychiatry*, 2009. 14(9): p. 865.
42. Rannals, M.D., et al., *Psychiatric Risk Gene Transcription Factor 4 Regulates Intrinsic Excitability of Prefrontal Neurons via Repression of SCN10a and KCNQ1*. *Neuron*, 2016. 90(1): p. 43-55.
43. Van Den Bossche, M.J., et al., *Identification of a CACNA2D4 deletion in late onset bipolar disorder patients and implications for the involvement of voltage-dependent calcium channels in psychiatric disorders*. *Am J Med Genet B Neuropsychiatr Genet*, 2012. 159B(4): p. 465-75.
44. Hawi, Z., et al., *The role of cadherin genes in five major psychiatric disorders: A literature update*. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2017.
45. Kushima, I., et al., *High-resolution copy number variation analysis of schizophrenia in Japan*. *Molecular psychiatry*, 2017. 22(3): p. 430-440.
46. Nakazawa, T., et al., *Differential gene expression profiles in neurons generated from lymphoblastoid B-cell line-derived iPS cells from monozygotic twin cases with treatment-resistant schizophrenia and discordant responses to clozapine*. *Schizophrenia Research*, 2017. 181(Supplement C): p. 75-82.
47. Smalley, M.J., et al., *Dishevelled (Dvl-2) activates canonical Wnt signalling in the absence of cytoplasmic puncta*. *Journal of Cell Science*, 2005. 118(22): p. 5279-5289.

48. Bousman, C.A., et al., Negative Symptoms of Psychosis Correlate with Gene Expression of the Wnt/ β -Catenin Signaling Pathway in Peripheral Blood. *Psychiatry Journal*, 2013. 2013: p. 852930.
49. Kang, J., H. Park, and E. Kim, IRSp53/BAIAP2 in dendritic spine development, NMDA receptor regulation, and psychiatric disorders. *Neuropharmacology*, 2016. 100(Supplement C): p. 27-39.
50. Fromer, M., et al., De novo mutations in schizophrenia implicate synaptic networks. *Nature*, 2014. 506(7487): p. 179-184.
51. Purcell, S.M., et al., A polygenic burden of rare disruptive mutations in schizophrenia. *Nature*, 2014. 506(7487): p. 185-190.
52. McKinney, B., et al., DNA methylation as a putative mechanism for reduced dendritic spine density in the superior temporal gyrus of subjects with schizophrenia. *Translational psychiatry*, 2017. 7(2): p. e1032.
53. Okazaki, S., et al., A novel nuclear protein with zinc fingers down-regulated during early mammalian cell differentiation. *Journal of Biological Chemistry*, 1994. 269(9): p. 6900-6907.
54. Alonso, M.B.D., et al., Identification and characterization of ZFP-57, a novel zinc finger transcription factor in the mammalian peripheral nervous system. *Journal of Biological Chemistry*, 2004. 279(24): p. 25653-25664.
55. Li, X., et al., A Maternal-Zygotic Effect Gene, Zfp57, Maintains Both Maternal and Paternal Imprints. *Developmental Cell*, 2008. 15(4): p. 547-557.
56. Chen, X.-S., et al., Advancements in the Underlying Pathogenesis of Schizophrenia: Implications of DNA Methylation in Glial Cells. *Frontiers in Cellular Neuroscience*, 2015. 9: p. 451.
57. Rivollier, F., et al., Methylomic changes in individuals with psychosis, prenatally exposed to endocrine disrupting compounds: Lessons from diethylstilbestrol. *PLOS ONE*, 2017. 12(4): p. e0174783.
58. Ladd-Acosta, C., et al., Common DNA methylation alterations in multiple brain regions in autism. *Molecular psychiatry*, 2014. 19(8): p. 862-871.
59. Chisholm, K., et al., The association between autism and schizophrenia spectrum disorders: A review of eight alternate models of co-occurrence. *Neuroscience & Biobehavioral Reviews*, 2015. 55: p. 173-183.

60. Rutten, B.P.F., et al., Longitudinal analyses of the DNA methylome in deployed military servicemen identify susceptibility loci for post-traumatic stress disorder. *Mol Psychiatry*, 2017.
61. Teroganova, N., et al., DNA methylation in peripheral tissue of schizophrenia and bipolar disorder: a systematic review. *BMC Genetics*, 2016. 17: p. 27.
62. Pidsley, R., et al., Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology*, 2014. 15(10): p. 483.
63. Lee, S.-A. and K.-C. Huang, Epigenetic profiling of human brain differential DNA methylation networks in schizophrenia. *BMC Medical Genomics*, 2016. 9(Suppl 3): p. 68.
64. Jaffe, A.E. and R.A. Irizarry, Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biology*, 2014. 15(2): p. R31.
65. Cerminara, N.L., et al., Redefining the cerebellar cortex as an assembly of non-uniform Purkinje cell microcircuits. *Nat Rev Neurosci*, 2015. 16(2): p. 79-93.
66. Hannon, E., et al., Interindividual methylomic variation across blood, cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric phenotypes. *Epigenetics*, 2015. 10(11): p. 1024-1032.

6 Memory restoring properties of a synthetic TrkB agonist

Abstract	p.175
Keywords	p.175
Introduction	p.176
Materials and Methods	p.177
Animals	p.177
Drug treatment	p.178
Object location task	p.178
Apparatus	p.178
Testing procedures	p.178
Y-maze	p.179
Statistical analyses	p.180
Results	p.180
Object location task	p.180
Y-maze spontaneous alternation test	p.181
Discussion	p.181
Conclusion	p.183
References	p.186
Supplementary Material	p.190
Methods	p.190
Results	p.191

Artemis Iatrou¹, Marion J.F. Levy^{1,2,3}, Nick van Goethem¹, Maxime Cazorla⁴,
Didier Rognan⁵, Harry W. Steinbusch¹, Laurence Lanfumey^{2,3}, Daniel L.A. van
den Hove^{1,6,*}, Gunter Kenis^{1,*}

1 School for Mental Health and Neuroscience (MHeNs), Department of Psychiatry
and Neuropsychology, Maastricht University, Maastricht, the Netherlands

2 Centre de Psychiatrie et Neurosciences, Inserm U 894, Paris, France

3 Université Paris Descartes, UMRS894 Paris, France

4 Grenoble Institute of Neuroscience, Grenoble, France

5 UMR7200 CNRS, Laboratoire d'Innovation Thérapeutique, Strasbourg, France

6 Laboratory of Translational Neuroscience, Department of Psychiatry,
Psychosomatics and Psychotherapy, University of Wuerzburg, Wuerzburg,
Germany

* These authors have contributed equally

Abstract

Enhancing the dysregulated brain-derived neurotrophic factor/tropomyosin-receptor-kinase B (BDNF/TrkB) signalling pathway has been suggested as a promising therapeutic strategy for restoring cognitive impairment in Alzheimer's disease (AD). A growing body of evidence supports that TrkB agonists attenuate and even reverse cognitive deficits associated with the progression of the disease in murine models of AD. In this study, we investigated the memory restoring action of TB001, a novel synthetic compound specifically designed to activate TrkB receptors, and we compared it to 7,8-dihydroxyflavone (DHF), a commonly tested but unspecific TrkB agonist. Our findings indicate that TB001 can rescue AD-related memory deficits *in vivo* on APP^{swe}/PSEN1^{dE9} mouse model of AD. As such, acute as well as chronic administration of TB001 was able to restore the genotype-dependent memory deterioration in the object location task. In contrast, solely chronic treatment with DHF was able to partly restore the observed memory deficits. Altogether, this study further advocates the use of TrkB agonists as treatment options for AD. Particularly, we show that TB001 displays promising therapeutic properties, characterized by cognitive restoring properties and a rather fast mechanism of action which makes it a good therapeutic alternative in order to alleviate the most common AD-related symptomatology.

Keywords: Alzheimer's disease; TrkB agonist; TB001; BDNF; DHF; Memory Restoration

Introduction

Alzheimer's disease (AD) is a chronic, neurodegenerative disease clinically associated with severe cognitive impairment. Its main hallmarks are the presence of oligomeric beta amyloid ($A\beta$), which form degradation-resistant aggregates termed plaques, and hyperphosphorylated tau protein that leads to the formation of intraneuronal neurofibrillary tangles (NFTs). Despite the large body of research dedicated to elucidate the underlying pathology and its aetiology, both still remain highly elusive, spurring the scientific community to find additional support for existing hypotheses and to generate new views on the pathogenesis of AD. With the passage of time, one of the most prevailing theories is that aggregation of $A\beta$, in a variety of forms, induces a molecular cascade leading to a calamitous sequence starting from synaptic dysfunction, synaptic loss, NFT formation and, ultimately, neuronal death [1]. Variations to this theory have been developed in order to explain the mechanisms underlying synaptic atrophy, and one of the most prominent hypotheses is related to disturbances in brain-derived neurotrophic factor (BDNF) / tyrosine receptor kinase B (TrkB) signalling [2]. In line with the neurotrophin hypothesis of AD, a dysregulated BDNF/TrkB signalling system could decrease neuronal resilience to hallmark insults, such as $A\beta$, concomitant with disintegrated synaptic structure and function.

BDNF is a neurotrophin, which, through the TrkB receptor, activates three main signalling pathways in order to induce cellular proliferation, differentiation and survival [3]. In both AD patients and mouse models of AD, BDNF has been shown to be dysregulated, i.e. generally downregulated either in the periphery or centrally [4]. Furthermore, delivery of BDNF in the brain exerts neuroprotective actions and partly restores cognitive deficits in diverse animal models of AD [5]. Accordingly, it has been suggested that BDNF-targeting treatments could have therapeutic potential for AD treatment. However, BDNF itself has a very poor pharmacologic profile characterized by a very short half-life and inability to cross the blood-brain barrier (BBB), hampering its direct application as a therapeutic agent [6, 7]. Loopholes to this problem have been provided by various research groups who have developed and tested molecules or synthetic compounds that can mimic the effect of BDNF.

One of the most studied compounds is 7,8-dihydroxyflavone (DHF). DHF is a flavonoid first described as an antioxidant but later characterized as a TrkB agonist with profound effects on TrkB activation [8]. Pharmacologically, while it penetrated the BBB it has a rather poor oral bioavailability [9, 10]. In murine models of AD, many studies showed a beneficial effect of DHF on memory performance [8, 11-16], although some failed to exhibit memory-restoring properties [17]. The inconsistent performance of DHF has raised questions in relation its mechanism of action and its specificity as a TrkB agonist.

In pursuit of a compound that could specifically activate TrkB, our collaborators at University of Strasbourg employed a structure-based *in silico* screening approach and manufactured a synthetic molecule, TB001 [18]. More specifically, a 3D pharmacophore was derived from the X-ray structure of the BDNF loop L4. Due to the specific binding nature of the compound as well as its mimicking of the BDNF structure, TB001 is a promising therapeutic candidate for disorders that would benefit from enhanced BDNF/TrkB signalling, among which AD.

Based on the convincing evidence endorsing a role of dysregulated BDNF/TrkB signalling in AD, as well as the design of small synthetic molecules with the potential to normalize this system, the present study focused on assessing TB001 as a therapeutic intervention in memory decline, particularly associated with AD. We thus aimed to evaluate the memory- restoring properties of TB001 and compared them to those of DHF. Spatial memory of a transgenic mouse model of AD, i.e. APP^{swe}/PSEN1^{dE9} (APP/PS1) mice, was assessed in acute and chronic treatment paradigms, with the use of the object location task (OLT). Furthermore, working memory post chronic compound administration was evaluated by means of the alternating version of the Y-maze.

Materials and Methods

Animals

All experimental animal research was executed according to protocols provided by the local Animal Ethical Committee of Maastricht University, Maastricht, the Netherlands (UM-DEC 2014-034) and governmental guidelines.

Five-month old APP/PS1 and wild-type (WT) C57BL/6J female mice, derived from an in-house breeding program (parental cohort: C57BL/6J and APP/PS1 purchased from Charles River, France and Jackson, ME, USA, respectively), were used. The double transgenic APP/PS1 line 85 carries a co-integrate of 1) chimeric human/mouse APP695 carrying the Swedish mutation (K594M/N595L) and 2) human PS1 with deletion of exon 9, each under control of a mouse prion protein promoter [19]. The APP695 Swedish mutation leads to a 2-3 fold overexpression of the APP protein, while the PS1^{dE9} mutation causes an increase in the production of A β 42 over A β 40, thereby leading to plaque deposition from 4 months of age [20]. The mice were housed according to their genotype in groups of 2-4 per ventilated standard Makrolon cage under standard conditions (22 °C, 60% humidity, 12:12h light/dark cycle [19:00 lights on], food and water *ad libitum*) and background noise provided by a radio. All experimental procedures were conducted during the animals' active phase. Weekly weight monitoring, and frequent coat state evaluations served as general

well-being indicators.

Drug treatment

At 5.5 months of age, the transgenic animals received daily i.p. injections of either TB001 (2 mg/kg), 7,8-DHF (DHF; 5 mg/kg) or their vehicle (2% Tween-80, 0.5% Tylose and 0.9% NaCl) [21], creating 6 experimental groups: WT-TB001 (N = 15), WT-DHF (N = 15), WT-Veh (N = 15), AD-TB001 (N = 12), AD-DHF (N = 12), AD-Veh (N = 9). Of note, DHF is soluble to organic solvents, and in literature protocols utilizing DMSO as solvent are most commonly encountered. On a previous study conducted by our group we observed DMSO-induced cognitive deficits (Sup. Fig.1) in naïve mice and therefore an alternative dissolution method was followed for this project. The administration period lasted for 30 days. The first injection was performed 1h prior to the first testing session of the OLT in order to assess the acute effects of the given compounds on memory consolidation processes. For the remainder of the administration period, the compounds were administered approximately 15h prior to any experimental procedure to exclude acute effects (see Fig.1 for the timeline of the behavioural batteries).

Object location task

Apparatus

The apparatus consisted of a polyvinyl chloride circular arena (diameter: 48 cm) with a 40 cm high wall. During all procedures, the testing room was dimly illuminated (8 lux). For each trial, two identical objects were placed in predefined positions as described below. The four different sets of objects used were: (1) a cone made of brass (maximal diameter 6 cm and total height 3.8 cm), (2) a transparent glass bottle (diameter 2.7 cm, height 8.5 cm) filled with sand and water, a massive metal cube (2.5 cm × 5 cm × 7.5 cm) with two holes (diameter 1.5 cm), and (4) a massive aluminium cube with a tapering top (4.5 cm × 4.5 cm × 8.5 cm) [22]. The weight of the objects made it impossible for the animals to displace them.

Testing procedures

Prior to testing, the mice were habituated to the experimenter, the injection protocol, the arena, the objects as well as the testing sessions. Each testing session consisted of two 4-minute trials, the familiarization (T1) and the probe trial (T2). During T1, the mouse was introduced into the arena, which contained 2 identical objects in fixed diametrical positions, by facing the middle of a wall. When the 4 min had passed, the mouse was placed back in its home cage. After the familiarization

trial, using predetermined inter-trial intervals (ITIs), T2 was conducted. At T2, the mouse was placed back into the arena in which one of the objects had shifted location. The novel location of the object was randomized in a balanced manner over the groups and the different sessions in order to reduce potential bias due to preference for a particular location [23]. The exploration time during T1 and T2 (E1 and E2, respectively) was recorded and analysed manually using video recordings. Exploratory behaviour was specifically defined as the direction of the nose at a distance no more than 1 cm and/or touching the object with the nose or front paws [23]. Any observed climbing or sitting on the object was not considered exploration. The olfactory cues from the arena and the objects were extinguished with 70% ethanol in between sessions. The exploration time at both sessions (E1, E2) was considered as a locomotion indicator as well as an indirect anxiety measurement. Cognitive performance was assessed with the discrimination index D2, which is defined as the ratio of the difference between the exploration time of the new and the old location, and the total exploration time during T2 (E2). Thus, this ratio indicates whether the mouse spent relatively more time in the novel location than the old, which is considered an indication of spatial memory. For all memory performance measurements, a comparison with no discrimination between the two objects, i.e. a D2 score of 0, was performed. A minimal total exploration time of 6 seconds was set as an inclusion criterion [23, 24]. Three intervals were tested at baseline, before treatment, i.e. 2h, 3h, and 4h, in order to identify the optimal ITI for the rest of the experimental period. Based on the results from the baseline study, a 3h ITI was used for the acute and chronic experiments.

Y-maze

Spatial working memory at APP/PS1 mice was assessed by using the Y-maze spontaneous alternation test, as previously described [24]. Each testing session consisted of a 5 min exploration trial. During this trial, the mouse was placed in the centre of the Y-maze and was free to explore the arena. The number as well as the order of the entries in the various arms was recorded. An entry was only registered when the mouse had all 4 paws placed in the arm. A successful triad was registered when the animal would visit all three different arms consecutively. The olfactory cues from the arena and the objects were extinguished with 70% ethanol in between sessions. The outcome parameter for working memory was the percentage of successful alternations the mouse performed and was calculated as the number of the 14 first triads divided by the total number of alternations (i.e. the total number of entries minus 2) $\times 100$. A percentage higher than 50% signifies functional working memory.

Statistical analyses

Two-way analysis of variance (ANOVA) was performed for comparison between groups accounting for the main and interactive effects of genotype (WT, APP/PS1) and treatment (vehicle, DHF, TB001). One-way ANOVA was performed for comparison among treatments (vehicle, DHF, TB001) within the APP/PS1 group. As indications for memory performance, a one-sample t-test was performed for the Y-maze spontaneous alternation test and the OLT, with reference values 50 and 0, respectively. Statistical significance was set at a α -level of 0.05. Statistical analyses were performed within the R statistical environment (version 3.3.2) and GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA).

Results

Object location task

At baseline, APP/PS1 mice showed higher exploration times during the first trial when compared to WT littermates (E1: $t(64) = 2.551$, $p < 0.05$) (data not shown). Repeated measures ANOVA on E2 of the OLT performed after chronic administration of the compounds revealed an overall effect of the genotype $\times$ treatment interaction ($F(2,56) = 6.3$, $p < 0.01$) (Table 1), indicating that the APP/PS1 mice tend to explore more the objects when treated with TB001 or DHF.

For assessing memory performance, the D2 scores were analyzed (Fig.1). The optimal ITI used for the acute and chronic version of the OLT was selected among three different intervals tested at baseline (2h, 3h, 4h; data not shown). At the 2h ITI, intact spatial memory was observed in all animals, whereas at a 4h ITI all groups had forgotten the original object location. Moreover, the 3h ITI was able to accurately discriminate between genotypes, i.e. displaying intact and impaired memory performance in WT and APP/PS1 mice, respectively, and, hence, was used for the experiments addressing the acute and chronic effects of the TrkB compounds. At baseline, using the 3h ITI, APP/PS1 mice performed significantly worse than WT mice ($t(55) = 2.594$, $p < 0.05$) and could clearly not distinguish between the novel and the old location of the object (one-sample t-test; $t(21) = 0.9062$, $p > 0.05$) (Fig.2A). The results of the acute version of the OLT showed that only vehicle-treated WT and TB001-treated APP/PS1 could distinguish the familiar and the novel location ($t(28) = 2.073$, $p < 0.05$ and $t(20) = 2.960$, $p < 0.01$, respectively; Fig.2B). After chronic administration of the compounds, WT mice treated with vehicle ($t(24) = 2.131$, $p < 0.05$) and DHF ($t(22) = 2.804$, $p < 0.05$), as well as the TB001-treated APP/PS1 mice ($t(17) = 2.714$, $p < 0.05$) were able to distinguish between the familiar and the novel

location of the object (Fig.2C). Thus, both acute and chronic administration of TB001 restores memory impairment in the APP/PS1 mice.

Y-maze spontaneous alternation test

Working memory was evaluated by comparing the percentage of alternations per group to chance level of 50% (Fig.3). It was shown that solely the TB001-treated mice performed significantly better than chance level ($t(\geq 10) \geq 2.815$, $p < 0.05$), suggesting functional working memory. Overall, no significant effects of genotype, treatment or genotype $\times$ treatment could be observed for the percentage of alternations. Additionally, no significant effects of genotype, treatment or genotype $\times$ treatment was observed for the number of arm entries.

Discussion

This study focused on evaluating the memory-restoring properties of TB001, a synthetic TrkB agonist, by means of a spatial and working memory assessment. Overall, our results highlight its memory restoring nature in a transgenic animal model of AD, APP/PS1 mice. Such observations further strengthen the notion postulated previously in the literature concerning the beneficial effects of regulating BDNF/TrkB signaling in AD. However, in contrast to the majority of the studies reporting on the unique memory-restoring effects of DHF, here, we particularly observe such effects with the administration of TB001.

The main outcome from this study is that TB001 can restore AD-associated memory deficits. In order to counteract the genotype-associated memory impairment, TB001 and DHF were administered and their acute and chronic action was evaluated. TB001 clearly attenuated spatial and working memory impairment in APP/PS1 mice both in acute and chronic conditions. Empirical research on synthetic TrkB agonist compounds, including DHF, has shown that they have an impact on enhancing and rescuing cognitive function. Specifically, *in vitro* work on DHF has indicated that this outcome is mediated by the effect of the compound on synaptic structure and plasticity [16, 25]. Nevertheless, the acute effects of such compounds are poorly studied. Previously, we had shown that acute administration of TB001 on naïve mice leads to enhancement of cognitive performance in a similar experimentation paradigm (Sup. Fig.). Here, we indicate that TB001 exerts its beneficial effects, even after one single administration, on APP/PS1 mice as well. Memory consolidation is executed by a two-step mechanism characterized by an early protein synthesis-independent step followed by a late (>3h) protein synthesis-dependent one [12]. Both phases are associated with synaptic plasticity, which is mediated partly by a BDNF

dependent mechanism, the hippocampal long-term potentiation (LTP) at the CA3-CA1 synapses [26]. The late-phase (L-LTP) is accompanied by changes in synaptic strength and by structural reorganisation of the actin cytoskeleton as well as protein synthesis [27]. Such activity-dependent structural plasticity has been found to be dependent on phosphorylation of the TrkB receptor [27]. Based on our experimental paradigm of acute administration, the effect of TB001 on memory performance is more likely due to priming of synaptic strength rather than molecular changes targeting AD-related pathological cascades. Thus, the acute beneficial effects observed in APP/PS1 mice reflect a symptomatic mechanism of actions rather than a pathology-therapeutic one. Overall, the results extracted from this experiment strongly advocate that administration of TB001 impacts on memory consolidation, thereby improving optimal cognitive functioning and restoring memory deficits associated with the pathophysiology of AD.

On the other hand, DHF showed less consistent results throughout. A single dose of DHF was not sufficient to rescue cognitive deficits in APP/PS1 mice. Further, a 4-week treatment of DHF seemed to restore memory function, although this effect did not reach statistical significance. While most studies attribute positive effects of DHF on memory function [11, 14], other, more recent ones did not present comparable results [17]. A closer look at the former studies indicates that DHF restored memory deficits in 12 to 15-month-old 5xFAD transgenic mice after 10 days and 4 months of administration at 5 mg/kg [10, 14, 16] as well as in a 3,5 to 5-month-old mouse model of neuronal loss after 2 weeks of administration using the same dose [13]. Additionally, another study assessing memory performance in the OLT showed a beneficial effect after a single administration of DHF at a dose of 0,1 mg/kg both in WT and APP/PS1 mice at 7 month of age using a 24h ITI [12]. However, in a different mouse model of AD, i.e. 4 to 6-week-old APP23/PS45 mice, DHF did not alleviate cognitive deficits after a 2-week treatment at 5 mg/kg [17]. Similarly, in 9 month old APP/PS1 mice, a single dose did not improve spatial memory impairment while a 30-day administration did rescue it [28]. Altogether, it appears that DHF has different effects depending on the mouse model as well as the way and duration of administration, which may explain the modest effect in the present study. Another difference between the aforementioned studies and ours is that we used female mice that have been reported to acquire the AD phenotype earlier than males of this APP/PS1 strain.

Another notion raised by our data is that both TrkB agonists, TB001 and DHF, may have anxiolytic properties on APP/PS1 mice. DHF has been previously reported to exert beneficial effect on anxiety-like mouse models [29-31]. Our assumption is made on the basis of the observed increase in E2 exploration time in treated AD groups in the chronic OLT paradigm. While this outcome measurement of

the OLT has not been formally validated as anxiety-related stress, it is commonly used to draw qualitative preliminary conclusions for the stress levels of the animals [32]. Therefore, chronic administration of TB001 and DHF could potentially have anxiolytic properties with effects discernible when applied on the disrupted BDNF/TrkB signaling system.

Conclusion

To date, the involvement of the BDNF/TrkB system in the pathogenesis of AD still remains elusive. Could impaired BDNF/TrkB signalling directly impact upon $A\beta$ formation leading to neurodegeneration or does it, indirectly, affect the resilience of neurons to amyloid or tau insults? Our study shows that molecules mimicking the action of BDNF have beneficial effects on cognitive functions in an animal model of AD. Here, TB001, a synthetic TrkB agonist, yields encouraging results as a memory restoring compound. When administered acutely, we support that the mechanism of action of TB001 is mainly symptomatic and possibly does not directly address the cause of AD. However the persistent effect on memory function after chronic administration connotes a more intricate mechanism of action, that should be elucidated in order to capitalize the use of TB001 to its full extent. En masse, TB001, exhibiting therapeutic properties against memory impairment, appears to be more effective than DHF, the most well-studied TrkB agonist.

The clinical potential and exploitation of TB001 as a novel treatment option for AD could be highly profitable. According to the outcomes of this study supplementary read-out parameters investigated suggest that single administration of TB001 can be considered free of side effects, as far as well-being, locomotion and anxiety-like behavior is concerned. Further, chronic administration could potentially elicit anxiolytic effects on the APP/PS1 mice, an effect that should be verified by designated behavioural batteries.

Overall, it can be concluded that TB001 is an appealing pharmacological candidate for regulating memory functions in AD. Further investigation on the underlying mechanisms of action in a molecular level will assist in better characterizing the molecule per se, but also optimize the mode and duration of administration. Finally, it cannot be excluded that TB001 has anxiolytic properties and thus its application on disorders such as depression and PTSD should be also addressed in perspective research lines.

Figure 1: Timeline of the experiment. Training for the object location task (OLT) started at 5 months of age (20 weeks) and the baseline data were obtained from week 21-23. Intraperitoneal (i.p.) administration with 7,8-dihydroxyflavone (DHF), TB001 or vehicle started at week 23, where acute measurements were reported, and continued for 4 weeks daily. At week 26-27 we investigated the effects of the chronic administration of the compounds on spatial and working memory, by means of the OLT and the Y maze spontaneous alternation test (Y maze alt).

Figure 2: Object location task (OLT) was used in order to evaluate the differential effect of the administered compounds (vehicle, 7,8-dihydroxyflavone (DHF), TB001) on spatial memory performance. Displayed are the D2 scores of the different treatment groups and genotypes throughout the different sessions of the OLT with a 3h intertrial interval (ITI). (A) Spatial memory performance evaluation between wild-type (WT) and AD mouse model (APP/PS1), N = 25-35 per genotype; Acute (A) and chronic (B) effects of the administered compounds on WT and APP/PS1 mice, N = 9-15 per treatment. Bars represent mean + S.E.M. per group; ^: Significantly different from 0 in one-sample t-test, $p < 0.05$; ^^: $p < 0.01$

Figure 3: Y maze spontaneous alternation test was used to evaluate the effects of the administered compounds (vehicle, 7,8-dihydroxyflavone (DHF), TB001) on working memory performance of wild-type (WT) and AD (APP/PS1) mice. Data are expressed as mean + S.E.M. ^: Significantly different from 50% in one-sample t-test, $p < 0.05$; ^^: $p < 0.01$.

Task		Parameter	Mice						Effect ANOVA
			WT vehicle	WT DHF	WT TB001	AD vehicle	AD DHF	AD TB001	
OLT	Acute	E2 (s)	23.35	23.04	18.6	15.07	19.75	24.06	$p > 0.05$
		S.E.M.	2.89	3.78	2.3	2.57	2.12	3.93	
	Chronic	E2 (s)	22.08	16.83	11.83	12.09	26.82	21.11	2x3 Genotype*Treatment $p < 0.05$
		S.E.M.	3.62	2.29	1.44	1.31	4.07	4.16	
Y maze	Alternating	Entries (#)	24.13	23.93	20.80	23.33	27.83	26.36	2x3 Genotype*Treatment $p > 0.05$
		S.E.M.	1.4	2	1.2	1.6	2.8	3	

Table 1: Mean, and S.E.M, of exploration times in the object location task (OLT) (E2: average exploration time T2) and number of entries the Y-maze. 2x3 Genotype*Treatment/ Genotype: significant genotype*treatment interaction/genotype effect in 2-way ANOVA. WT, wild-type; AD, Alzheimer's disease mouse model; DHF, 7,8-dihydroxyflavone

References

1. Mattson, M.P., Pathways towards and away from Alzheimer's disease. *Nature*, 2004. 430(7000): p. 631.
2. Song, J.-H., J.-T. Yu, and L. Tan, Brain-derived neurotrophic factor in Alzheimer's disease: risk, mechanisms, and therapy. *Molecular neurobiology*, 2015. 52(3): p. 1477-1493.
3. Boule, F., et al., Epigenetic regulation of the BDNF gene: implications for psychiatric disorders. *Mol Psychiatry*, 2012. 17(6): p. 584-96.
4. Peng, S., et al., Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *Journal of neurochemistry*, 2005. 93(6): p. 1412-1421.
5. Nagahara, A.H., et al., Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med*, 2009. 15(3): p. 331-7.
6. Pardridge, W.M., Blood-brain barrier delivery. *Drug discovery today*, 2007. 12(1-2): p. 54-61.
7. Pardridge, W.M., D. Wu, and T. Sakane, Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. *Pharmaceutical research*, 1998. 15(4): p. 576-582.
8. Liu, X., et al., A synthetic 7, 8-dihydroxyflavone derivative promotes neurogenesis and exhibits potent antidepressant effect. *Journal of medicinal chemistry*, 2010. 53(23): p. 8274-8286.
9. Liu, C., C.B. Chan, and K. Ye, 7,8-dihydroxyflavone, a small molecular TrkB agonist, is useful for treating various BDNF-implicated human disorders. *Translational Neurodegeneration*, 2016. 5: p. 2.
10. Chen, C., et al., The prodrug of 7, 8-dihydroxyflavone development and therapeutic efficacy for treating Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 2018: p. 201718683.
11. Andero, R., et al., 7,8-dihydroxyflavone, a TrkB receptor agonist, blocks long-term spatial memory impairment caused by immobilization stress in rats. *Hippocampus*, 2012. 22(3): p. 399-408.
12. Bollen, E., et al., 7, 8-Dihydroxyflavone improves memory consolidation processes in rats and mice. *Behavioural brain research*, 2013. 257: p. 8-12.

13. Castello, N.A., et al., 7,8-Dihydroxyflavone, a small molecule TrkB agonist, improves spatial memory and increases thin spine density in a mouse model of Alzheimer disease-like neuronal loss. *PLoS One*, 2014. 9(3): p. e91453.
14. Devi, L. and M. Ohno, 7,8-dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of Alzheimer's disease. *Neuropsychopharmacology*, 2012. 37(2): p. 434-44.
15. Yang, Y.-J., et al., Small-molecule TrkB agonist 7, 8-dihydroxyflavone reverses cognitive and synaptic plasticity deficits in a rat model of schizophrenia. *Pharmacology Biochemistry and Behavior*, 2014. 122: p. 30-36.
16. Zhang, Z., et al., 7, 8-dihydroxyflavone prevents synaptic loss and memory deficits in a mouse model of Alzheimer's disease. *Neuropsychopharmacology*, 2014. 39(3): p. 638-650.
17. Zhou, W., et al., No significant effect of 7, 8-dihydroxyflavone on APP processing and Alzheimer-associated phenotypes. *Current Alzheimer Research*, 2015. 12(1): p. 47-52.
18. Cazorla, M., et al., Identification of a low-molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. *The Journal of clinical investigation*, 2011. 121(5): p. 1846.
19. Jankowsky, J.L., et al., Mutant presenilins specifically elevate the levels of the 42 residue β -amyloid peptide in vivo: evidence for augmentation of a 42-specific γ secretase. *Human molecular genetics*, 2003. 13(2): p. 159-170.
20. Garcia-Alloza, M., et al., Characterization of amyloid deposition in the APP^{swe}/PS1^{dE9} mouse model of Alzheimer disease. *Neurobiology of disease*, 2006. 24(3): p. 516-524.
21. Bollen, E., et al., Improved long-term memory via enhancing cGMP-PKG signaling requires cAMP-PKA signaling. *Neuropsychopharmacology*, 2014. 39(11): p. 2497.
22. Chadwick, W., et al., Amitriptyline-mediated cognitive enhancement in aged 3 $\times$ Tg Alzheimer's disease mice is associated with neurogenesis and neurotrophic activity. *PLoS One*, 2011. 6(6): p. e21660.
23. Sierksma, A.S., et al., Chronic phosphodiesterase type 2 inhibition improves memory in the APP^{swe}/PS1^{dE9} mouse model of Alzheimer's disease. *Neuropharmacology*, 2013. 64: p. 124-136.

24. Akkerman, S., et al., Object recognition testing: methodological considerations on exploration and discrimination measures. *Behav Brain Res*, 2012. 232(2): p. 335-47.
25. Zeng, Y., et al., 7, 8-dihydroxyflavone rescues spatial memory and synaptic plasticity in cognitively impaired aged rats. *Journal of neurochemistry*, 2012. 122(4): p. 800-811.
26. Leal, G., C.R. Bramham, and C.B. Duarte, Chapter Eight - BDNF and Hippocampal Synaptic Plasticity, in *Vitamins and Hormones*, G. Litwack, Editor. 2017, Academic Press. p. 153-195.
27. Lai, K.-O., et al., TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory. *Nature neuroscience*, 2012. 15(11): p. 1506.
28. Gao, L., et al., TrkB activation by 7, 8-dihydroxyflavone increases synapse AMPA subunits and ameliorates spatial memory deficits in a mouse model of Alzheimer's disease. *Journal of neurochemistry*, 2016. 136(3): p. 620-636.
29. Andero, R., et al., Effect of 7,8-Dihydroxyflavone, a Small-Molecule TrkB Agonist, on Emotional Learning. *The American journal of psychiatry*, 2011. 168(2): p. 163-172.
30. Shirayama, Y., et al., Alterations in brain-derived neurotrophic factor (BDNF) and its precursor proBDNF in the brain regions of a learned helplessness rat model and the antidepressant effects of a TrkB agonist and antagonist. *Eur Neuropsychopharmacol*, 2015. 25(12): p. 2449-58.
31. Zhang, J.-c., et al., Antidepressant effects of TrkB ligands on depression-like behavior and dendritic changes in mice after inflammation. *International Journal of Neuropsychopharmacology*, 2015. 18(4): p. pyu077.
32. Morellini, F., Spatial memory tasks in rodents: what do they model? *Cell and tissue research*, 2013. 354(1): p. 273-286.
33. Callaghan, C.K. and Á.M. Kelly, Neurotrophins play differential roles in short and long-term recognition memory. *Neurobiology of learning and memory*, 2013. 104: p. 39-48.
34. Mizuno, M., et al., Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. *Journal of Neuroscience*, 2000. 20(18): p. 7116-7121.

35. Cirulli, F., et al., Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. *Hippocampus*, 2004. 14(7): p. 802-807.
36. Santos, N.C., et al., Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochemical pharmacology*, 2003. 65(7): p. 1035-1041.
37. Hanslick, J.L., et al., Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. *Neurobiology of disease*, 2009. 34(1): p. 1-10.
38. Lebedev, M.A., et al., Representation of attended versus remembered locations in prefrontal cortex. *PLoS biology*, 2004. 2(11): p. e365.

Supplementary Material

Prior to the study presented in this paragraph, an experimental paradigm was set up in order to assess the cognitive enhancing properties of TB001 on naïve mice. While the two studies are independent, findings from the latter provided guidelines for the design of the main project presented here and thus will be briefly described.

Methods

Animals

All procedures involving animals and their care were conducted in conformity with their institutional guidelines that are in compliance with nation and international laws and policies (Council directive 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé; permissions 006269 to L.L.).

C57BL/6J male mice (Charles River Laboratories, l'Abresle, France) were used. Upon arrival to the laboratory, the mice were housed in groups of four per cage under standard conditions (22 °C, 60% humidity, 12:12h light/dark cycle [19:00 lights on], food and water ad libitum) and background noise provided by a radio, for 1 week prior to any experimental procedure. Experiments were performed with 2 month old mice (12-13-week-old). All experimental procedures were conducted during the animals' active phase. Weekly weight monitoring, and frequent coat state evaluations served as general well-being indicators.

Drug administration

At 2 months of age, WT mice received a single intraperitoneal (i.p.) injection of TB001 (2 mg/kg), ANA-12 (TrkB antagonist; 0.5 mg/kg) and vehicle solutions, subsequently creating 4 experimental groups: the two experimental groups receiving either TB001 (TB, N = 20) or ANA-12 (in 1% DMSO, 1% Tween-80, 0.9% NaCl; ANA, N = 20; (Maybridge, Thermo Fisher Scientific, Illkirch, France) and their respective control groups receiving TB001 vehicle injections (SAL, N = 20), the second control group receiving ANA-12 vehicle (1% DMSO, 1% Tween-80, 0.9% NaCl; DMSO, N = 20). The administration paradigm followed a crossover experimental design with an injection interval based on the washout period of the drug, known from preliminary screening data. Therefore all animals received by the end of the experiment four injections of different solutions

(Vehicles, TB001, ANA-12). The injection was performed 1 hour prior to the first testing session of the OLT, in order to target the acquisition phase of memory and evaluate its impact on general memory performance as tested by the OLT as described above.

Statistical analysis

The exploration times were similar across conditions during the T1-familiarization session (E1; $F(3,284) = 1.68$, $P = 0.17$). Similarly, a two-way ANOVA on exploratory behaviour during the T2- testing session (E2) revealed no significant effect of either treatment ($F(3, 201) = 1.37$, $p = 0.25$), ITI ($F(2,201) = 0.27$, $p = 0.78$) or interaction ($F(6,201) = 0.9$, $p = 0.5$) (Only average E1 and E2 data shown in Table 2).

Results

Two-way ANOVA on the D2 scores in the 1h, 4h and 24h ITI showed a significant effect of treatment ($F(3,18) = 11.22$, $p < 0.01$, Supplementary Fig. 1). This effect was explained by the 4h ITI, where Bonferroni's multiple comparisons revealed significantly better performance of the TB001-treated group in comparison with its control ($t = 3.03$, $p < 0.05$). Detailed analysis of the D2 scores overtime indicated that administration of saline and ANA-12 does not have an impact on hippocampus-dependent memory performance among the intervals. Additionally, the performance of these groups follows a normal delay-dependent "forgetting"-like curve. On the contrary, administration of 1% DMSO tends to affect the D2 scores overtime (Bonferroni's multiple comparisons: $t_{1h-24h} = 2.04$, $p > 0.05$, Supplementary Fig. 1). Administration of TB001 tends to enhance cognitive performance from 1h to 4h interval (Bonferroni's multiple comparisons: $t_{1h-4h} = 1.9$, $P = 0.07$), which returns to baseline in the 24h testing session. Thus, this effect results in a unique cognitive performance curve that reaches its peak at 4h ITI in comparison with the rest of the groups that perform the best in the 1h ITI. Focusing on the 4h interval, we observed that the TB001 treated animals perform significantly better than the controls (one-way ANOVA followed by Bonferroni's multiple comparisons: $t(3,60) = 2.87$, $p < 0.05$). Finally we examined whether the different groups can distinguish between the old and the novel location, by comparing their D2 scores with 0. We observed that solely the saline and TB001-treated animals succeed in recognizing the novel location throughout all the intervals. The DMSO-treated group could only distinguish between the two objects in the 1h interval.

The results revealed procognitive and memory enhancing properties of TB001 *in vivo*, in healthy C57BL/6J mice. A single i.p. injection (2 mg/kg) significantly improved spatial memory performance in the OLT, 5 hours post-administration. This finding further supports the key-role of BDNF in memory formation that has been stated in existing literature. It has been repeatedly reported that the BDNF mRNA and protein upregulation as well as phosphorylation of target-molecules in the BDNF/TrkB signaling pathway in memory-associated areas is a primary outcome of learning and memory procedures in different stages such as acquisition, early and late consolidation [33]. Negative manipulation of the levels of BDNF by means of intracerebral infusion of antisense BDNF oligonucleotides was shown to impair spatial memory performance [34]. On the same tone, intrahippocampal administration of BDNF improves spatial memory performance [35]. TB001 appears to be the only TrkB agonist with spatial procognitive properties on naïve mice, detectable 5 hours after an acute injection. Additionally, our results suggest that the effect of TB001 treatment prior to acquisition is reflected during the early memory consolidation stage. Therefore, it is highly recommended that future experimental set-ups focus on different administration paradigms focusing on different time points and their effect on diverse memory stages.

Our study also revealed an interesting effect of DMSO on cognitive performance. In fact, the DMSO-treated animals were not able to distinguish between the old and the novel object location, at all intertrial intervals. These findings further advocate the hypothesis that administration of DMSO is relatively risky. Yet, DMSO is the most common solvent for *in vivo* administration of hydrophobic chemical compounds [36]. An extensive study of Hanslick on the effect of DMSO on the developing murine CNS revealed that it induced widespread apoptosis associated with dramatic elevation of caspase 3-positive neurons, at all ages tested [37]. To this end, the dose of DMSO used in this study, but also in our own paradigm (1%; 0.3 ml/kg), was reported to induce 150% more apoptotic neurons than the control in 4 weeks old mice, 8 hours post-administration. Complementary post mortem analysis revealed that the neurodegeneration was restricted to cortical regions and the white matter. Another study further examined the effect of biologically innocuous concentration of DMSO by means of electrophysiology, where they showed that incubation of murine brain slices in DMSO significantly changed the intrinsic neuronal excitability especially within the pyramidal neurons of the CA1 area of the hippocampus, a main region associated with cognitive functioning. Extrapolating these observations back to our study, we support that DMSO-treated animals were not able to perform equally to the saline group due to presumed deleterious effects of DMSO on prefrontal cortical neuronal networks, associated with attention and working memory consolidation [38], and/or hippocampal neuronal connectivity. Subsequently, henceforth in our experimental pipeline, DMSO was avoided as a solvent and alternatively 2% Tween-80, 0.5% Tylose and 0.9% NaCl a validated solvent solution, was used [21].

Task		Parameter	Mice				Effect ANOVA
			WT sal	WT DMSO	WT TB001	WT ANA	
OLT	Acute	E2 (s)	26.18	29.77	26.95	27.77	p > 0.05
		S.E.M.	1.90	2.66	2.25	2.14	

Supplementary Table 1: Mean, and S.E.M, of exploration times in the object location task (OLT) (E2: average exploration time T2). WT, wild-type; sal, vehicle; DMSO, dihydroxy sulfoxide; ANA, ANA-12

Supplementary Figure 1: Object location task (OLT) was used in order to evaluate the differential effect of the administered compounds (vehicle (SAL), TB001, ANA-12, dimethyl sulfoxide (DMSO)) on spatial memory performance. Displayed are the D2 scores of the different groups throughout the intervals of the OLT sessions. N = 16 mice/ group. Bars represent mean + S.E.M. per group; *: Significant difference at Bonferroni multiple comparisons, $p < 0.05$; ^: Significantly different from 0 in one sample t-test, $p < 0.05$; ^^: $p < 0.01$; ^^: $p < 0.001$.

7 General conclusion

Strengths and Limitations

p.198

Future Perspectives

p.201

The prevalence of mental and neurodegenerative disorders definitely constitutes a hurdle in the global contemporary progress and evolution. Brain-related disorders like Alzheimer's disease (AD), schizophrenia (SZ) and bipolar disorder (BP) represent a ticking bomb under worldwide, and primarily Europe's and Northern America's, economy; a bomb difficult to be dismantled due to the increasing aging population and the lack of efficient treatment strategies. In numbers, the annual expenses of 2015 dedicated to AD research, healthcare and long-term care for 44 million patients were estimated to approximately \$605 billion worldwide, a number that was estimated to reach \$1 trillion by this year. Concerning mental disorders, in 2004 it was estimated that the annual economical burden of schizophrenia alone was \$41 billion and was predicted to increase with a rate of \$2.6 billion per year, which would mean that currently it has surpassed \$1.3 billion. As distressing as these numbers are, they

cannot be compared to the devastating personal impact of these disorders per se. While all three disorders have distinct clinical manifestations, every single one of them deprives the afflicted individuals of their humane nature, converting them into highly dependent beings or social pariahs. The readily prescribed therapeutic treatments are mainly symptomatic and fail to battle against the aetiology of the diseases, which are overall highly unknown. And even when administered, the social stigma that follows the patients hinders even more the recovery. In order to face this societal emergency and vindicate the quality of life of these patients, the efforts of the scientific community should intensify in order to elucidate the aetiopathological underpinnings of these three diseases. This thesis, with a fresh and innovative perspective on disease mechanisms, contributes to this effort by examining the epigenetic mechanisms in relation to these three disorders.

Right from the start, at **CHAPTER 1**, the question of the heritability gap in mental and neurodegenerative disorders is posed, and my assumption that epigenetic mechanisms might be able to fill it in drives the rest of this thesis. A brief introduction on the epigenetic machinery is provided with a special focus on the most studied DNA modifications, DNA methylation and hydroxymethylation. Once the role of epigenetic regulation is explicitly stated, it becomes apparent that dysregulation at the epigenetic level could have devastating repercussions on the molecular and cellular balance and thus lead to pathological conditions. In light of this notion, three pathological disorders - AD, SZ and BP- are highlighted and aberrant methylomic and hydroxymethylomic patterns are reviewed.

After this prelude, a thorough review follows in **CHAPTER 2**, starting with a timeline of epigenetic technology spanning from the first ever targeted antibody-based techniques to state-of-the-art genome-wide arrays and sequencing methodologies, and how this technology has been capitalized for AD research. Through the use of epigenomic technology, it is now possible to e.g. examine epigenetics beyond DNA methylation and CpG-specific methylation, as well as to profile cross-regional and cross-tissue epigenetic variation in health and disease, with issues such as cell-type heterogeneity been resolved either with wet lab or bioinformatics approaches. However, it is suggested that only if all potential from epigenomic technology is exploited in brain structures relevant to the incipient stages of AD pathology, breakthroughs in therapeutic and diagnostic research fields will be achieved. To this end, I illustrate all empirical evidence on how AD pathology is reported to stem from brainstem nuclei and I strongly encourage a deeper examination of epigenetic signatures in the LC and the DRN especially at prodromal stages of the disease.

Following up on this notion, **CHAPTER 3** presents the first study to interrogate the DNA methylome and hydroxymethylome of the brainstem in AD. Using bisulfite (BS)- and oxidative bisulfite (oxBS)-treated DNA as input, three cytosines states were characterized, i.e. unmodified, methylated and hydroxymethylated cytosine. A unique (hydroxy)methylomic profile is hence revealed in the DRN with the course of the disease, as described by Braak stage progression. Strikingly, most of the identified epigenetic signatures, while not being reported in previous EWAS studies in different AD-pathology afflicted brain structures, have been described in the literature to be associated with A β pathology. Such observation suggests that the two pathological hallmarks of AD are entwined with epigenetic modifications as the disorder advances. Overall, this study demonstrates an innovative way of discriminating three states of cytosines and its application in AD research where distinct signatures can be observed.

In **CHAPTER 4**, another epigenetic mechanism is studied in relation to AD diagnosis, i.e. that of microRNAs (miRs). Based on the fundamental role of miR-137 in neuronal function, its involvement in AD was tested and, more specifically,

potential methylomic variation was set under investigation. MIR137 methylation and expression patterns were studied in human post mortem tissue from the mediotemporal gyrus, a brain structure already affected by AD pathology in relatively early stages. In addition to a case-control comparison, these two readout parameters were also correlated with beta amyloid and tau pathology load. Additionally, in an attempt to bring this research line one step closer to the clinics, the blood methylation profile of miR-137 was also examined in a longitudinal cohort that included healthy participants of which some converted to AD patients over a 4.5 year time period. The outcomes exhibit that a specific genomic locus neighboring MIR137 tends to be differentially methylated with disease diagnosis in the periphery and furthermore it is highly associated with both beta amyloid and tau load centrally. While expression changes are not reported here, a novel observation is raised concerning the stability of miR-137 as post mortem interval severely affects its decay rate. Hence, this study suggests that methylation changes in the blood of AD patients should be closely interrogated as they could potentially serve as disease biomarkers. On a side note, it is obliquely highlighted that miR-137 has a rather fast turnover profile and consequently research lines investigating expression changes should be cautious and consider the post mortem interval as a covariate to any analysis pipeline.

Expanding the spectrum of brain disorders to neuropsychiatric illnesses, **CHAPTER 5** examined psychosis- as well as disease-specific cerebellar methylation signatures in two independent cohorts, which served as discovery and replication cohort. No global methylation changes were discovered in schizophrenia and bipolar disorder, either when studied together as major psychosis disorder or in isolation, in either of the cohorts. Next, differentially methylated positions (DMPs) and regions (DMRs) were identified in the discovery cohort and two regions within the ZFP57 and PM20D1 genetic loci were significantly hypermethylated in both SZ and BP. The significance of this finding is further strengthened by the fact that the ZFP57 was found to be hypermethylated in BP at the replication cohort as well. These findings reinforce the hypothesis that epigenetic changes play a critical role in the pathophysiology of neuropsychiatric disorders and advocate an important role for the cerebellum in these conditions.

CHAPTER 6, while still dedicated to the pathophysiology of AD, deviates from the epigenetics-centered character of this thesis and presents an in vivo attempt to pharmacologically restore memory impairment, the main symptom of AD. TB001, a synthetic compound that activates the BDNF/TrkB signaling system, a highly dysregulated system in AD, is examined as a potential therapeutic alternative, in order to rescue memory deficits in a mouse model of AD. Excitingly, the main observation of this study is that the specific compound not only improves cognitive performance when administered both acutely and chronically, but also outperforms DHF, the most-studied compound with a similar mechanism of action.

Strengths and Limitations

The work presented in this dissertation is a collection of studies with a common denominator; the interrogation of epigenetic signatures characteristic of disease pathology or diagnosis. A major strong point of the projects presented here is the methodology used to approach the relevant research questions. Two out of four studies leverage state-of-the-art epigenomics technology, namely the Infinium MethylationEPIC array, to tackle questions previously interrogated with its predecessor, Infinium Human Methylation 450K BeadChip array. To our knowledge these studies represent the first application of the EPIC technology to mental and neurodegenerative disorders and our results set the pillars for more thorough yet affordable interrogation of DNA modifications in pathological conditions. Furthermore, the study conducted and described in chapter 2, represents the first ever examination of the hydroxymethylome with the EPIC array platform. The combination of an inventive way of pre-processing DNA before loading on the beadchip with a platform that provides double the coverage of the most commonly used one, is a unique way of distinguishing true methylation from hydroxymethylation, and a chief asset of this dissertation. Complementary to the value of dealing with highly burdensome disorders, this thesis further provides alternative perspectives of approaching the pathology. It endeavours to study unconventional brain structures, not widely studied in the relevant disorders, i.e. AD-brainstem, SZ/BP-cerebellum, and unravel unique signatures previously overlooked due to the trending research lines.

Notwithstanding the aforementioned strengths, this dissertation is also subjected to some general as well as more specific limitations. The most common and persistent limitation of epigenetic studies is the small to moderate sample size, which results in limited power of the study to detect significant changes. This is particularly problematic when it comes to the detection of DMPs. An additional issue related to DMP detection is the determination of a significance threshold for declaring a CpG site as differentially methylated, taking multiple testing into account, as 10^{-7} seems to be rather conservative. This is exemplified in this thesis by the results of the EWAS showing that while approximately 8% of the tested probes pass nominal significance threshold, they fail to reach genome-wide significance levels after multiple testing correction. A loophole to this issue could be provided via the development of novel statistical correction tools. Such bioinformatics approaches, adjusted for EWAS studies, could for instance take into consideration the *cis* correlation among probes harbouring the same genetic locus and disregard these corrections. Alternatively, as mentioned in chapter 2, a way to strengthen and substantiate results from one study, is to perform replication studies, or exploit publically available data that have used the same methodologies and conduct meta-analyses.

With the advent of affordable Illumina technology, the Infinium Human Methylation 450K Beadarray and its successor, Infinium MethylationEPIC have been established as the workhorse of epigenetic research lines worldwide. Thus, the common use of datasets among different groups has been facilitated, allowing for replication studies, as in chapter 6, as well as meta-analyses. However, 450k and EPIC technology is not limitation-free. While the probes on the chip are annotated for most of the known genes, they only substitute a small fraction of all CpGs in the genome. Moreover, they only cover CpGs, leaving non-cytosine methylation uninvestigated. Therefore, designating studies utilizing this technology as “genome-wide” is rather inaccurate, as the genomic coverage is restricted to 1.5% of the CpG sites across the human genome. The EPIC array used in chapters 3 and 6 comes with improved coverage, especially at enhancer sites, but still maintains the CpG restricted features of the 450K array. Therefore, the use of methylation sequencing technology should be endorsed on perspective epigenetic research lines in order to obtain a wider coverage and thus a deeper understanding of disease-related methylomic profiles.

A major strength that followed the advancement of epigenomic technology is the detection of a “true” DNA methylation signal, achieved by the addition of an extra oxidative step at the bisulfite conversion, as described in chapter 2 and applied in chapter 3. Before the use of the oxBS conversion step, solely the combined DNA methylation and hydroxymethylation signal could be extracted. Nowadays, the two signals can be distinguished. To this extent, it should be stressed that previously reported results, utilizing regular BS conversion technologies, with unsegregated signals, should be revisited and interpreted with caution. Yet again, the oxBS approach does not take into consideration DNA formulation and carboxylation, whose signal is now interlaced with the hydroxymethylation one. The low presence of 5-formylcytosine and 5-carboxylcytosine in the brain make the aforementioned issue a minor limitation. What constitutes a larger problem is the fact that analysis methodologies do not progress with the same pace as the development of the technology and to date only indirect approaches for determining DNA hydroxymethylation levels are used. As mentioned in chapter 3, the approach followed, i.e. subtracting the oxBS from the BS signal, due to noise and technical variation results in negative 5-hmC values, which are biologically meaningless. In an attempt to exclude these values, strict detection thresholds are implemented, resulting in dropping approximately 40% of the probes on the BeadChip.

Another consideration is that research lines treat methylation and hydroxymethylation analyses equally, while these two modifications apart from distinct biological roles also appear with different abundance in the brain. To this end, current analytical pipelines tend to disdain the latter. This can be exemplified from the analysis followed for differentially hydroxymethylated regions (DHR) determination in chapter 3. The single DHR that emerged from our data suggest that spatial correlation analysis for differentially hydroxymethylated positions (DHP) might need to be optimized as hydroxymethylation per se is present but not as enriched as methylation in brain tissue and the sliding window might need to be adjusted.

Two more limitations emerge from the EWAS performed in this thesis. Firstly, the WGCNA analysis employed in chapter 3 was conceptualized in order to create networks of co-expressed genes, and has not yet be optimized for either multiple-probes-one-gene data, like EPIC array datasets, or data of such length/volume (~340k probes). Secondly, the cellular heterogeneity of the bulk cell populations used as input produces differential signals from i.e. neurons and glial cells, potentially confounding the outcomes from the analysis followed, an issue also mentioned in chapter 2. Deconvolution approaches for cell type heterogeneity have been developed, in order to infer the underlying cell type proportions of the tissue under interrogation. However, reference-based algorithms are only available for the dorsolateral prefrontal cortex (DLPFC) on human brain material, and in this thesis the tissue used for EWAS was from the brainstem and the cerebellum, structures very different from each other, but, most importantly, profoundly different from the DLPFC. In an attempt to overcome this limitation, surrogate variable analysis was employed in order to capture patterns of variation due to any unmodeled factors. While valid, other more accurate methods able to define cell type composition of brain tissue should be developed.

The majority of aforementioned limitations will soon be resolved with the progress of bioinformatics procedures, but the question of the biological relevance of epigenetic research lines persists. This research era is governed by reductionism; scientists tend to deconstruct complex multidimensional questions with the hopes that once each element has become implicit, then it would be easier to connect the pieces and provide the complete picture of i.e. of a complex disease. However, extreme reductionism has shifted the focus from global clarity towards exhaustive attention on single cellular pathways, or even single genes of interest and thus creating hypotheses of "one gene, one phenotype". Few are the disorders though that follow strict Mendelian heritability paradigms and that indeed one gene is responsible for one phenotype. In more complex disorders, it appears to be more common that an extensive network of molecular players is responsible. And yet, since the keen scientific eye finds it hard to conceive the magnitude of the neuronal processes and networks, it still resorts on the oversimplified notion that drivers of common diseases are either exclusively common or collectively rare alleles.

To this end, the loophole of epigenetics was offered to the scientific enterprise to fill in these gaps and add an extra level of complexity. But are epigenetic mechanisms treated once again with a reductionism approach: waiting to be given detrimental connotations in relation to diseases, either they have functional outcomes or not? In order to avoid such evolution of the epigenetic research, gene set enrichment analyses as well as pathway construction approaches or networks of co-methylated probes by means of weighted correlation gene analysis (WGCNA) should be followed, allowing sets of genes with altered epigenetic regulation to be identified like in chapter 3 and chapter 6, and further on be functionally characterized. Additionally, integrative genomics, transcriptomics and epigenomics approaches as suggested in chapter 2 can provide clarity on the complex pathophysiology of common disorders.

Finally, a sign of our times and the biggest limitation that the research is facing is publication bias. The dearth of funding as well as professional ambition have prompted researcher to submit mainly positive results for publication, as they are more likely to be published and cited. This has led to an unforeseen level of publication bias, topped with non-replicable studies as well as retraction of multiple articles from high impact journals due to scientific misconduct and/or incorrect data analysis. Therefore, I would like to underline that the lack of “conventionally” positive results, as seen in chapter 4, should not discourage the researcher to publish the outcomes of the study or to join forces with other studies in performing meta-analyses. Such action would not only save time and resources for fellow scientist with similar research queries but would also contribute in resolving a major issue that the scientific community is facing: the skewness of scientific literature by ignoring negative results.

Future Perspectives

Exciting times lie ahead, as all the aforementioned limitations can work as tinder for kindling of novel ideas and breakthroughs. Affordable genome-wide technology in combination with the exponential increase in high quality brain tissue available from brain banks worldwide will probably reinforce studies with larger sample size. Until then, approaches utilizing independent cohorts in order to replicate findings can be employed to strengthen observations. Furthermore, truly genome-wide approaches by means of sequencing will be highly beneficial for the field, especially if applied on a single-cell basis. However, it shall not be overlooked that the technological breakthroughs should be accompanied by advancements in related statistical tools to handle the generated data that will serve as a backbone. Thus, dry lab techniques based on computational modelling and machine learning that are gaining momentum will assist in processing as well as taking full advantage of the generated data. Most importantly, in order to provide answers to the aetiology and pathophysiology of a disease and thus advance

towards developing accurate diagnostic and therapeutic tools, research lines should direct their attention towards integrating genomics and epigenomics. By employing relevant approaches such as Mendelian randomization (MR) and methylation quantitative trait loci (meQTL), described in chapter 2, the interplay among genetic variation, epigenetic modifications and environmental factors could be elucidated, contributing to bridging the heritability gap. Ultimately, answers to whether epigenetics are the cause or consequence of complex diseases could be provided and tested using state-of-the-art epigenetic editing methodologies.

Overall, studies like the ones presented in this dissertation, following fundamental research lines with novel approaches, provide more insight into the pathological mechanisms that characterize complex diseases. Moreover they set the pillars for perspective functional work in order to clearly identify the effect of the identified epigenetic signatures in the aetiology and/or progression of the pathology of AD, SZ and BP. More importantly, the datasets and results generated here contribute to the worldwide epigenetic data collection of each disease. Thus, my work assists in an intensive and collaborative effort of the scientific community to structure multilevel causal networks of each disease in order to predict personalized resilience or vulnerability for disease pathology, and potentially identification of disease biomarkers as well as novel targets for therapeutic drug development.

8 Summary

The work presented in this thesis collectively examines the involvement of epigenetic mechanisms in mental and neurological disorders, in particular Alzheimer's disease (AD), schizophrenia (SZ) and bipolar disorder (BP). The epigenetic mechanisms under interrogation are primarily DNA modifications, and to a lesser extend microRNAs (miRNAs).

CHAPTER 1 of this dissertation familiarizes the reader with DNA modifications and the status quo of their involvement in the pathology and pathogenesis of these specific neurological disorders. After this concise introduction, **CHAPTER 2** unfolds the timeline of epigenomic technology advancements, starting from the earliest antibody-based methods and following up to contemporary genome-wide arrays as well as sequencing approaches, in the context of AD. While the reported technological progress is highly remarkable, it does not come limitation-free and therefore limitations as well as suggestions to overcome them are also reported. Then, the reader is challenged with the question whether the "state-of-the-art" technology, used for AD research, is actually capitalized optimally for the discovery of novel therapeutic/preventive and diagnostic strategies. The personal, yet rational answer provided, suggests that for a pharmacological option to be effective, it should be administered at the most incipient stages of the disease in order to target the preclinical pathology and therefore prevent the later seemingly unresponsive to treatment phase. Considerable evidence suggests that early dysregulation in the brainstem, more specifically in the raphe nuclei and the locus coeruleus, accounts for the earliest, non-cognitive, symptomatology, indicating a potential causal relationship with the pathogenesis of AD, and thus evidence associating these two nuclei with AD is reviewed. The take-home message from this chapter is that temporal and spatial manifestations of the disease should be aligned and, thus, structures more closely related to the prodromal stages of AD should be examined further. Capitalizing the advanced and nowadays affordable epigenomics technology, the pairing of brainstem pathology with deviant epigenetic regulation could yield novel candidate targets for the development of early biomarkers as well as therapeutic alternatives that could halt or even reverse the deleterious progression of AD.

Following on that hypothesis, **CHAPTER 3**, the first experimental study, sets the dorsal raphe nucleus (DRN) as the cornerstone for interrogating early-stage AD epigenetic signatures. Specific genome-wide patterns of methylated (5mC), hydroxymethylated (5hmC) as well as unmodified (UC) cytosines were investigated by means of the Illumina MethylationEPIC array, in relation to disease progression as characterized by Braak staging. Using an inventive approach, genomic DNA extracted from DRN homogenates was treated in parallel with bisulfite and oxidative bisulfite, allowing for distinction of the true methylation signal from the combined methylation and hydroxymethylation one, and thus permitting the examination of three discrete cytosine states. Differentially modified and unmodified positions as well as regions emerged which were then interrogated at a systems-level by employing a weighted gene co-expression network analysis (WGCNA), correlating co-methylated probes with disease progression in order to create modules of probes with a significant involvement in pathology aggravation. Finally, a pathway analysis was performed for the most significant modules for each cytosine state subset in order to reveal which biological pathways are associated with aggravation of AD pathology. Interestingly, the reported modified and unmodified genomic positions and regions have never been identified in epigenome-wide studies previously, but the genes affected have previously been shown to be involved in the pathophysiology of AD, specifically with respect to beta amyloid pathology. This study represents the first systematic epigenetic analysis, distinguishing among 5mC, 5hmC and UC in the DRN of AD patients making use of EPIC array technology. To this end, a unique structure-specific epigenomic profile in association with Braak stage progression was identified.

CHAPTER 4, while remaining focused on examining epigenetic variation in AD, deviates from the previous line and sets microRNAs (miRNAs) in the centre of the attention. MiR-137 regulates presynaptic vesicle release as well as synaptic strength within the adult hippocampus and its expression is regulated by genetic factors as well as epigenetic modifications. The connecting link between miR-137 and AD has been previously detected in AD-associated downregulated MIR137 expression, centrally as well as peripherally, and, furthermore, in cognitive deficits associated with rare genetic variants of MIR137. Thus, this chapter aimed to investigate the interplay between methylation and expression of MIR137 in relation to AD diagnosis and pathology in brain and blood samples. Making use of Illumina's BeadChip and 450K Beadarray, expression and methylation patterns of MIR137 were profiled, respectively, in brains of AD patients and age-matched controls. Additionally, increasing the clinical relevance of this project, miR-137 blood methylation changes over time were interrogated in a

cohort of healthy aged individuals, some of which converted to AD patients during MIR137 tends to be, it was highly associated with pathological hallmarks of AD. On a side note, it was also demonstrated that MIR137 expression is highly affected by the post mortem interval (PMI) of provided tissue, indicating that the mRNA decay rate of the specific miR-137 is rather fast, baring into consideration that the PMI was less than 4h. Collectively, the reported MIR137 methylation patterns are correlated with AD pathology centrally and there is a tendency towards decreased global methylation of MIR137 with AD diagnosis peripherally. The work presented in this chapter is the first of its kind to examine the methylomic profile of MIR137 in the brain and blood of AD patients, and further advocates that methylation signatures in the blood could potentially serve as disease biomarkers.

The experimental pipeline of **CHAPTER 5** focused on detecting methylomic signatures printed in the cerebellum of patients with major psychosis, as defined by the diagnosis of schizophrenia (SZ) or bipolar disorder (BP), and replicate them in an independent cohort. The widely overlooked cerebellum was the centre of attention in this chapter as psychosis-associated cognitive and emotional dysfunctionalities have been attributed to it. We therefore set to examine methylation changes associated with major psychosis as an umbrella term, and then proceed to identify whether these modifications are more closely related to SZ or BP. Utilizing once more the Illumina technology (EPIC array) we did not manage to identify cytosine methylation differences between controls and psychotic patients. Expanding our analysis to regional methylation analysis we detected two regions at the ZFP57 and PM20D1 loci to be significantly hypermethylated in both SZ and BP. Following up on this observation, we acquired the data from an independent epigenome-wide study and applied the same pipeline on them in order to replicate the aforementioned results. To this end, the region associated with ZFP57 emerged solely as a BP-related methylation mark, hinting that the specific locus is more closely associated with the pathophysiology of BP. Our study represents the first analysis of epigenetic variation associated with major psychosis leveraging the EPIC technology. To this end, it highlights that the BP-afflicted cerebellum is imprinted with distinct methylation signatures, a finding consistent between two independent cohorts.

The final experimental part of this thesis is presented in **CHAPTER 6** which deviates from the epigenetic-centred character of this dissertation while still focusing on AD pathology. This study represents an attempt to provide a therapeutic alternative to the main clinical symptom of AD, memory impairment. A pharmacological approach was followed targeting the brain-derived neurotrophic factor/

tropomyosin-receptor-kinase B (BDNF/TrkB) signalling pathway, which is highly dysregulated in AD. To this end we treated a transgenic mouse model of AD with TB001, a synthetic compound that activates the BDNF/TrkB signalling system, and we compared its efficacy with the most-studied compound with a similar, yet less specific, mechanism of action, 7,8 dihydroxyflavone (DHF). Our findings indicated that TB001 can rescue AD-related memory deficits in vivo upon acute as well as chronic administration. Excitingly, the specific compound outperformed DHF, which exhibited partly memory-restoring properties only after chronic treatment.

Overall, this dissertation approached fundamental complex disease-associated research questions from novel perspectives as well as highly innovative methodologies, and provided more insight into the underlying pathological mechanisms. Therefore, it contributed in an intensive and collaborative effort of the scientific community to structure multilevel causal networks of these disorders. Such networks will assist the prediction of personalized resilience or vulnerability for disease pathology, and potentially the identification of disease biomarkers as well as novel targets for therapeutic drug development.

9 Valorization

“What is your research about?” they ask me.

“Alzheimer’s disease” I respond.

“Ah great! We will definitely need you in a couple of years!” they say in a tone mingling excitement and fear for the future.

This is one of the most common reactions I receive once I try to explain what I do for a living to my relatives.

My relatives: middle-aged common people with mostly office jobs receiving input of a wide gamut of diseases that might affect them in the coming years, and amidst them Alzheimer’s disease, the seemingly most terrifying one. These people put their hope in individuals like me in order to treat a disease that could gradually erase their lifetime memories and their timeline thus far.

Me: a researcher on my 4th year of working experience attempting to gain a little more insight in the widely undiscovered world of epigenetics and complex diseases through a computer screen and huge datasets or animal experiments. The aforementioned reaction does not put me at ease, as I am aware that should any of them develop Alzheimer’s disease in the next decades, there may be nothing we can do; the time bomb has most likely been set to go off already and although help is on the way, it may be too late for them.

Currently, on average, for every day of an Alzheimer’s disease researcher’s life, 16,437 people are being diagnosed with the disease, which translates in a devastating sum of 6 million new cases annually, worldwide, adding an immense burden on families, caregivers and financial costs for society [1]. Should Alzheimer’s disease get eradicated, annual savings from payments for all American dementia patients could pay off the Greek government debt [2]. Similarly, burdensome is the scourge of mental illnesses with almost 40% of the developed countries population being diagnosed with a mental disorder, which corresponds to approximately 164.8 million people affected [3]. The need for a therapy is imperative. Nevertheless, this intense global scientific scouting around for treatment strategies has not reported back that many encouraging results.

While it is a fact that the lack of effective therapeutics is mainly due to the overly complicated nature of such disorders, one could also claim that the majority of research lines are restricted to pathological observations made decades ago and researchers seem rather skeptical to leap towards more innovative hypotheses. Scientists supported that their arsenal was lying within the genetic code, and expected that upon decoding the whole human genome in 2003, answers concerning causality and aetiopathogenesis would emerge. However, they soon realized that complex disorders, such as Alzheimer's disease, schizophrenia, bipolar disorder, are not of genetic origin only, but to a big extend depend on the impact of the environment, which plays an important role in their onset and progression. From then on, the field of epigenetics burgeoned and, thus, empirical basis was set for the speculative nature of the implication and mechanisms of epigenetic modification on disease pathogenesis. Therefore one might wonder: "could it be that for the last 4 decades the scientific world has been fighting the wrong adversaries?".

The work presented in this dissertation attempts to respond to this question by examining "unusual suspects" among players of disease pathology and pathogenesis. These "unusual suspects" are not only restricted to epigenetic mechanisms, but also extend to brain structures with previously underrecognized roles in disease pathogenesis and pathology. Most possibly this book will not have a directly observable impact on society or economy, but it is highly relevant for the academic enterprise. The majority of the chapters represent an exploratory body of work applied on human tissue specimens that could serve as pillars and thus maneuver future studies towards novel directions.

To this end, the research presented in **CHAPTER 3** is an interrogation of specific epigenetic modifications associated with Alzheimer's disease. The impact of this work on the scientific world lies in two levels. Firstly, being among the first of its kind to simultaneously examine three cytosine states, it does indicate that methylation and hydroxymethylation marks highly differ even in the same genetic locus and thus advocates that previously reported studies on methylation which looked into the combined signal of methylation and hydroxymethylation should be reviewed with caution. Secondly, in retrospect, one could claim that the conception of this study is highly relevant for society due to the novel view in a different brain structure. In contrast to the majority of the hippocampus/memory deficits-targeted research lines, the hypothesis driving this project is tailored to the most incipient preclinical disease manifestations that a patient experiences, and therefore considers the understudied brainstem structures [4]. While such study requires replication in order to highlight the validity of the outcomes, it might nominate novel targets, relevant to earlier stages of the disease, that can be used for diagnostic or treatment alternatives in future studies.

Similarly, the work presented in **CHAPTER 5** is an effort to define epigenetic signatures associated with mental disorders, namely schizophrenia and bipolar disorder. The novelty of this study lays in the interrogation of epigenetic marks in the understudied cerebellum. And indeed its impact is rather big as, regardless the small sample size of the cohort, the most differentially methylated regions were replicated in an independent cohort. Further mechanistic approaches are required in order to functionally characterize the meaning of this epigenetic signature at a systems level as well as cross-regionally in the brain with the ultimate goal of using the identified markers as preventive or diagnostic tools.

In the interim, the stature of epigenome-wide studies like the ones presented in **CHAPTER 3** and **5** is the broadening of knowledge until a more holistic understanding of the pathological underpinnings of these diseases is reached. Novel approaches covering previously underrecognized brain areas while employing internationally used methodologies, namely the Illumina arrays, add one more brick to this global effort to comprehend the width and depth of the biological mechanisms making an individual susceptible to Alzheimer's disease, schizophrenia or bipolar disease.

The project presented in **CHAPTER 4** does not solely focus on brain material, but on blood as well, which makes it more clinically relevant as well as more possible to have a societal impact. The blood is an easy accessible platform to study epigenetic changes throughout the lifespan and thus attempt to model epigenetic marker fluctuations during the onset and progression of a disease. Furthermore, blood markers could also be indicative of central events related with the outbreak of the pathology. To this end, changes in the i.e. methylation state of a genetic locus, or, to be more realistic, a combination of them, could indicate the conversion of a healthy person to an AD patient and therefore a simple blood test could serve as a diagnostic and/or prognostic tool.

The scientific work concluding this dissertation in **CHAPTER 6** tackles the main clinical symptom of Alzheimer's disease, memory loss, with the assistance of animal models. Excitingly, the administered compound that targets the BDNF/TrkB signaling system, managed to restore the memory impairment in a transgenic mouse model of Alzheimer's disease. However, what is the impact of this outcome? Perusing the literature, every week, another article gets published suggesting to have cured Alzheimer's disease in animal models. Still, regardless this tremendous 'success' rate, no effective, disease-eradicating treatment has been introduced to the market yet [5]. Under no circumstances should the importance of animal models be undermined, as they are the impetus of fundamental research, but yet again, the current animal models fail to portray the disease in an accurate and global manner, crashing the hopes for the successful translation to the human

situation. At this point I would like to clarify that intervention studies like this one cannot provide a shortcut to the cure. However, the significance of this research line lies in the fact that modulation of a fundamental signaling pathway seems to have beneficial effects in memory performance and therefore adds one more piece to the puzzle of the pathophysiology of Alzheimer's disease.

So upon reflection of the impact my work will have in various levels, I stay puzzled. The environmental stimuli that could influence the vulnerability of our genome increase per decade, with the ever-changing pace of life. Worldwide, people of similar disciplines make a change for the humankind by finding cures for the most common disorders. The lifespan thus is extended; but is the quality of the 30-40 prolonged years optimal? What society demands from scientists is a solution for all these three disorders, in the form of cure or prevention, as soon as possible. Eventually, holistic knowledge of epigenetics will offer novel alternatives to therapeutic approaches and/or fast and accurate diagnosis close enough to the onset of each disease. Hopefully by that time, personalized medicine will have progressed to the point that e.g. targeted epigenetic engineering will allow for an intervention that could cure the disease. Will this dissertation yield any therapeutic candidates? Wishfully yes. Of course it will not happen from one day to the next. Fundamental research is vital to set the pillars for applied research and to inspire researchers, generate novel ideas that eventually will lead to scientific breakthroughs. And for now, the fact that my research offers another stepping-stone towards better understanding of the complex nature of the disorders makes all the efforts worthwhile.

References

1. Association, A.s., 2018 ALZHEIMER'S DISEASE FACTS AND FIGURES, in *Alzheimers Dement.* 2018. p. 367-429.
2. Statista. Greece: National debt from 2012 to 2022 (in billion U.S. dollars). 2018, April; Available from: <https://www.statista.com/statistics/270409/national-debt-of-greece/>.
3. Wittchen, H.-U., et al., The size and burden of mental disorders and other disorders of the brain in Europe 2010. *European neuropsychopharmacology*, 2011. 21(9): p. 655-679.
4. Simic, G., et al., Does Alzheimer's disease begin in the brainstem? *Neuropathol Appl Neurobiol*, 2009. 35(6): p. 532-54.
5. Franco, R. and A. Cedazo-Minguez, Successful therapies for Alzheimer's disease: why so many in animal models and none in humans? *Frontiers in pharmacology*, 2014. 5: p. 146.

10 Curriculum Vitae

Artemis Iatrou was born on April 6th 1990 in Athens, Greece. Her path towards becoming a scientist commenced nearly a decade ago with her enrolment in the Bachelor degree programme in Biology in Patras, Greece (2008). Among the broad spectrum of fundamental biological knowledge she received during the 4 years of her Bachelor studies, neuroscience captured her interest and spurred her to apply for the Fundamental Neuroscience track of the Cognitive and Clinical Neuroscience Research Master in Maastricht University, The Netherlands (2012). For the compulsory 9-month internship of the aforementioned programme, under the distal supervision of Dr. Gunter Kenis and Dr. Daniël van den Hove, she joined the research team of Prof. Laurence Lanfumeu at the National Institute of Health and Medical Research (INSERM) in Paris, France (2013), where she was assigned a project aimed at the investigation of the role of neurotrophic signaling in Alzheimer's Disease (AD)-like in vitro and in vivo models. After successfully completing her Research Master studies (2014) top of her class, she obtained an NWO Graduate School of Translational Neuroscience fellowship in the same year to fund a PhD position supervised by Prof. Bart Rutten, Dr. Daniël van den Hove and Dr. Gunter Kenis. On the first year of her PhD she was involved in the EPIAD project, a European initiative focusing on interrogating the epigenetic signatures within the brainstem in AD (<http://www.epi-ad.eu/>), where she assisted in drafting the grant application with Dr. Daniël van den Hove. Fascinated by the field of epigenetics, she deviated from her classical wet lab practice and received training in bioinformatics during her one-year fellowship in one of the world-leading groups in the field, that of Prof. Jonathan Mill in the University of Exeter Medical School, UK (2016). Chasing expertise and not stability, she further applied and got funded for a second fellowship in order to gain more insight into the (epi)genetic editing techniques at the group of Prof. Manel Esteller, at IDIBELL, Barcelona (2017). Her work has been presented in various national and international conferences, including the Dutch Neuroscience meeting and Society for Neuroscience Annual meeting. Beyond her clear scientific interest, her drive to stay in Academia ignited her interest to plan and supervise practicals for Bachelor's and Master's students, tutor block courses for biomedical students but also take the role of PhD representative of the Neuroscience division. Currently, Artemis is focusing on exceling her culinary skills pending her departure for Chicago where she will start her post-doc at the RUSH Alzheimer's Disease Center.

MY PROFESSIONAL CAREER (2008-2018)

Published

Iatrou, A., Kenis, G., Rutten, B. P. F., Lunnon, K., & van den Hove, D. L. A. (2016). Epigenetic dysregulation of brainstem nuclei in the pathogenesis of Alzheimer's disease: looking in the correct place at the right time?. *CMLS*, 1-15

Lardenoije R., van den Hove D.L.A., Vaessen T.S.J., Iatrou A., Meuwissen K.P.V., van Hagen B.T.J. , Kenis G., Steinbusch H.W.M., Schmitz C., Rutten B.P.F., Epigenetic modifications in mouse cerebellar Purkinje cells: effects of aging, caloric restriction, and overexpression of superoxide dismutase 1 on 5-methylcytosine and 5-hydroxymethylcytosine, *Neurobiol. Aging*, Volume 36, Issue 11, November 2015, Pages 3079-3089

Lardenoije R., Iatrou A., Kenis G., Kompotis K., Steinbusch H.W.M., Mastroeni D., Coleman P., Lemere C.A., Hof P.R., van den Hove D.L.A., Rutten B.P.F., The epigenetics of aging and neurodegeneration, *Prog. Neurobiol.*, Volume 131, August 2015, Pages 21-64

Submitted and in preparation

M. Devall, E.L. Dempster, R.G. Smith, J. Burrage, A. Iatrou, E. Hannon, A.R. Smith, C. Troakes, S. Al-Sarraj, L. Schalkwyk, J. Mill, M. Weedon, K. Lunnon, Genome-wide characterization of mitochondrial DNA methylation reveals sex-specific patterns of genomic regulation in human brain

Submitted Neurobiol. Aging

R. Lardenoije*, J.A.Y. Roubroeks*, E. Pishva*, M. Leber, H. Wagner, A. Iatrou, A.R. Smith, R.G. Smith, L.M.T. Eijssen, L. Kleinedam, A.Kawalia, P. Hoffmann, T. Luck, S. Riedel-Heller, F. Jessen, W. Maier, M. Wagner, R. Hurlemann, G. Kenis, M. Ali, A. del Sol, D. Mastroeni, E. Delvaux, P.D. Coleman, J. Mill, B.P.F. Rutten, K. Lunnon, A. Ramirez*, D.L.A. van den Hove*, The Alzheimer's disease DNA (hydroxy)methylome

* The authors had equal contribution in the study

Submitted Mol. Psychiatry

A. Iatrou*, E. Pishva*, R. Lardenoije, J.A.Y. Roubroeks, J. Burrage, B.P.F. Rutten, J. Mill, G. Kenis, K. Lunnon, D.L.A. van den Hove, Methyloomic and hydroxymethyloomic profiling of the dorsal raphe nucleus in Alzheimer's Disease

* The authors had equal contribution in the study

In preparation

A. Iatrou, R. Lardenoije, J. A. Y. Roubroeks, E. Pishva, D. Mastroeni, P. D. Coleman, P.J., A. Ramirez, B. P. F. Rutten, G. Kenis, D. L. A. van den Hove, Epigenetic variation of the miR-137 gene in Alzheimer's disease

In preparation

A. Iatrou, M.J.F. Levy, A. Muurmans, N. van Goethem, M.Cazorla, D. Rognan, L. Lanfumey, D.L.A. van den Hove, G. Kenis. Neuroprotective and Memory Restoring Properties of TB001, a Novel Synthetic TrkB Agonist, in an AD Mouse Model

In preparation

A. Iatrou, E.L. Dempster, E. Hannon, E. Pishva, R. Lardenoije, J. Burrage, B.P.F. Rutten, G. Kenis, D. L.A. van den Hove, J. Mill. On the epigenetic theory of major psychosis: schizophrenia- and bipolar disorder- associated methyloomic signatures in the cerebellum

In preparation

12 Acknowledgments

I could not start this section without thanking my promotion team, Prof. dr. Bart Rutten, Dr. Daniel van den Hove, Dr. Gunter Kenis, who helped me and supported me throughout the long and windy road towards obtaining this degree and more importantly indicating to me what kind of researcher I want to be.

Daniel: one man, too many mottos.

You made me realize that indeed science is fun, and by reminding me every Monday at our meetings that every new week offers new chances (aw well challenges) you urged me to go the extra (s)mile in order to look for the truth!

Thank you for letting me open up my wings, teaching me how to fly and become a cosmopolitan researcher. Your faith and trust in my abilities was always there, even in the darkest hours. And in these darkest hours, your qualities as an exceptional human being shone through making me feel that all will be alright and a new day will dawn that the burden will be a bit less on my back. And that's all it takes to become a role model. You are my role model, the person I look up to and the person that inspires me to become the best researcher and supervisor I can and never have a day that I don't want to go to work. You are a true inspiration. Your romantic yet radical ideas about science as well as your passion should be the standard way of thinking in these highly turbulent times of research that we go through. Your humour, compassion, concern and care should be used as standard operating protocol for any senior faculty member that wishes to be an exemplary supervisor. For all the faith and effort you put in me, but also for all everyday things we have experienced the past 5 years, I am truly grateful and thankful.

Gunter, you are the person that possibly knows me the best from my promotion team. Back in 2012, we met for the first time as you were assigned to be my mentor during the Fundamental Neuroscience Research Master course. Regardless my highly hangover state (to this day I deeply wish that you hadn't realised), I immediately felt that you are the kind of person that I could share all my scientific and philosophical concerns and indeed not only did we have stimulating conversations but you also helped me develop critical and associative

thinking. By suggesting the internship to Paris (you, “Maybe you cannot go back home for Christmas this winter”, me “Who wouldn’t love to spend Christmas in Paris?”) you gave me a ticket in the TNP group firstly as a master student attendant and later on as a PhD candidate, and to this date I firmly believe that it is one of the best choices I ever made. Our relationship matured with time, but you always remained my mentor. You are the person I will first turn to when a challenge comes up, in personal or professional ground. Your devotion in research, your wittiness, your sense of humour and your values as a researcher are values that after half a decade of knowing you are deeply instilled in me and always guide me. Appreciation and thankfulness are too little of words to express my feelings for what you have offered to me!

A very special gratitude goes out to you Bart as well for assisting me and providing the funding for my PhD. My time abroad might have hampered frequent contact with you but nevertheless I deeply appreciate your enthusiasm and input at challenges I faced during the last semester of my PhD. You were always easily accessible anytime I needed assistance for personal and professional matters and for that “bridging the gap” I sincerely thank you.

A special mention and thank you is also addressed to Prof. Harry Steinbusch who initially granted me my scholarship and stood by me during the first year of my PhD and gave me the unique opportunity to attend the Society for Neuroscience and Brightfocus workshop at the infancy of my PhD, igniting even further my interest for Alzheimer’s Disease. Your quick visits in the office and our small chats were very illuminating for my research!

I would also like to express my special thanks to Prof. Laurence Lanfumey, Dr. Katie Lunnon and Dr. Raúl Delgado-Morales for hosting me at their lab, supervising and training me during my various internships.

Now apart from my official promotion team I would like to show my heartfelt appreciation to the people who added the tools to my skillset and formed me as a researcher throughout these years. Many thanks goes to the technician

team, Hellen, Marjan and Denise, that not only facilitated the admin processes but also assisted me in general wet lab and animal experimentation procedures. Additionally I would like to thank all the members of the CPV that helped me with my animal experiments. I owe a very important debt to Barbie that with her patience, generous support and exceptional attitude shaped and sharpened my wet lab skills during my “hands on” time of the PhD. Further, I would really like to thank you for your ever present smile in the corridor and your exciting words about my progress. Now, I would also like to salute Ehsan and Roy, for heroically tolerating my computational inquiries and always being willing and eager to teach me more being an endless source of inspiration. From the international front and in a chronological order, I would similarly like to thank Emilien, Vincent and Marion for their invaluable comments and tutoring in Paris, Adam, Jo, Bex and Eilis for the enormous help and training in Exeter, and last but not least Edi and Humberto for introducing me to the world of CRISPR and never roll your eyes when I was addressing yet another question towards you in Barcelona.

Finally, a big shout-out to the rest of the people that contributed at the very end and helped with the realisation of this book: Rachele, Andreas and Yorgos. Rachele, without you I would have cracked somewhere between finding dates and dealing with bureaucracy! Thank you so much for preventing that! Γκουλιώτα, σε ευχαριστώ πολύ για όλο τον χρόνο και την ενέργεια σου, και επιπλέον για την κατανόηση και δημιουργικότητα στην περίοδο της σύγχυσής μου! Γιώργο, ως κυριος συντελεστής της δημιουργίας αυτού του βιβλίου, θα ήθελα να σε ευχαριστήσω απο καρδιάς για όλη τη βοήθεια σου με το layout του βιβλίου.

These were the people that contributed significantly in the execution and finalization of this book. However, it goes without saying that the people that kept my mind sane and my spirit high are the unsung heroes of this dissertation. The following rather informal format of the acknowledgements is dedicated to these people. In these 6 years since I moved out of my motherland, I lived in seventh heaven so many times, but I also lost the earth beneath my feet a couple other. Through thick and thin these people came and shone their light and showed me the way.

It all started with the Fundamental Neuroscience research master year of 2014. The excellent selection criteria of Prof. Jos Prickaerts (Kudos Jos!) brought together a rather peculiar collection of people that up to these days I feel honored to have known and to have shared these 1.5-years with them. Ulisses, Karolina, Kim,

Astrid, Nynke, MJ, Bram, Beatrice, Danae thank you for all the nice memories. Simone, look at you! You made it! From Italian Casanova cycling to Belgium every night listening to Heroes and watching the usual suspects, you are now a most certainly well rounded scientist and more importantly a father. Good luck with everything that is to come your way! Dila, the ever-hippie! I cherish all our talks, adventures, cooking ventures every day and I am more than grateful to have met such a free spirit! I really hope that our paths will meet more frequently! Robert, you freaking genius! Where do I start with you and where do I end? In a sense you taught me so much about life in such a short time. The nonsense you talked always made sense to me, and the life you lead was like an adventure I was living by proxy (semi-proxy in Versailles). I weirdly thank you for the injections of extremity in my life and showing me how to think outside the box. Gerben, I could never have predicted our friendship to evolve like that but eij, here we are 6 years later! I have a very dear place in my heart for you. Thank you for all the support throughout the years. There will always be a cold frappe and toasty waiting for you at Anavissos, and of course always a hug to make you feel uncomfortable! Britto, if there is a person that has been next to me in my worst, this is you. Either at Coyote Ugly Saloon making a fool out of myself, or at the real hardships of life you were there or I want you to know that I am sincerely grateful for that.

The year in Paris followed and I was once more lucky to work with an amazing group of people. Mathilde, Vincent, Adeleine, Emilien, Said, Eleni, Fabien thank you so much for making me feel like home regardless my very poor French vocabulary. Apart from the excellent collaboration in the lab, you instilled into me good colleague practice by including me at the Boule and the after work world cup drinks. The most valuable thing I obtained in Paris though was getting to know Marion. How much goodness and nerdiness can you fit in such a petite person?! The way you stood next to me, tutored me, trained me, made me feel at home was priceless. And then back again in Maastricht: the drinking nights, the Halloween nights, the Star Wars nights, the Back to the Future nights, the Game of Thrones nights, the raclette nights...so many nice memories that I could have only had with you! Thank you so much!

And well, back again in Maastricht for the official start of the PhD. I was very lucky to be part of the coolest group in the department, the TNP group (or whatever it is called nowadays). From the 1.0 to the 2.0 I had an absolutely amazing time with you guys.

Nicole, while our time in Maastricht only shortly coincided, I had a very pleasant time with you at various extracurricular occasions. Your drive is really inspiring and your smile is very heart-warming. Welcome back to Maastricht! Mark, you weird specimen of a man! Your name carries so many trademarks: CrossFit, avocados, mama's food, California dreaming and so on. In retrospect it was a delight being your office mate! Hope that the Down Under treats you well! Magda, I still remember the first day that I met you and we went for vegetarian burgers. From the first time your directness, criticism and constructive complaints draw me to you and you were always a shelter for me to express my points of view about professional life and myself in general. Thank you for all the great talks and who knows...we might see each other at the States? Johana, you are fun! A special shout out to the time in Barcelona where we got to learn each other a bit more. Good luck with all that is following! Lars, my internships here and there never really allowed us to collaborate but during the TNParties I was absolutely flabbergasted by you. The energy you have in you is remarkable and so is your knowledge about whiskeys and beers! Kudos sir! Alix, thanks for letting us have a taste of Canadian kindness! Lotta. Girl if only we would have met some time ago...we would run Maastricht! Our social impulse was unparalleled! You cannot imagine how revitalizing our few meetings were. Thank you for that! Laurence, you are among the most kind, considerate and helpful people I have met in Maastricht. In my humble opinion you are such an encouraging example of a balanced successful female researcher. I am pretty sure you are going to do great things in this department. Ehsan. So funny isn't it? You were the guy that was meeting with Gunter before me during my Master's and you turned out to be a friend, a mentor and a consultant all progressively throughout these 4 years. Your capricious views on personal and professional terrains leave their mark and seem to be working very well for you! I truly enjoyed our attempts to reinstate the Friday drinks that ended up pouring our life stories over lukewarm beer. It was a bliss discussing our cultural background at Duke's with Jameson's, and having weekly potlucks. Thank you for all these moments. I can only wish you the best of luck to what is due to come! Janou! I still believe that you were created from icing sugar in a bakery. What a delight are you to have around...from whiskey cravings at 3am to whiskey tasting at noon. My souvenirs from our time together taste like cake, smell like any Islay whiskey and sound like Queen, or maybe Beatles. I had the most memorable times with you in the lab (the cell culture was our concert hall), or outside...in pubs, in tar barrel festivals, in Gilmore Girls nights, in pub quizzes, in cider drinking occasions, in BuzzFeed quizzes (during breaks only!), at Nando's, in Exeter, in Maastricht. It was a bliss being next to you through the good times and the challenging ones and may our friendship survive the distance!

The first year of the PhD...these were the days! The most amazing dream team that ever existed in the department that embraced me rather fast and made my time during and after work most memorable. This is when I started loving Maastricht! Your departure hallmarked the end of a chapter in this department. For all the memories and adventures I salute you ex comrades of UNS50!

Alejandro, we might have co-existed for a couple of days but your friendly vibe captivated me and still does after our meeting in NY! Looking forward to seeing you again in SF! Gisela! From stranger to friend in less than a day; what an amazing personality you have! Maarten, through ups and downs, even if we did not have the most compatible personalities I would be lying if I said that you did not mark the department and Maastricht in general! Nick. I am absolutely fortunate to have been the recipient of your kindness, generosity, work-life and real-life input as well as your humour throughout the years. We are on this rollercoaster of life and it is a hell of a ride! Thanks for tagging along! Sandra, let's see...what an intimidating persona! I am mostly happy that I was among the few that managed to sneak peek through your wall and see what I believe to be the real Sandra. I deeply appreciate our complaining sessions and I am extremely happy that you found your way to less turbulent environments where you can lay back and be valued personally and professionally. Joao, you are the first person I met even before my arrival to Maastricht. Without your help and support in societal and professional issues, I would be lost throughout the first semester of my time in the Netherlands. I cannot thank you enough. Now in your final form, the professor one, I deeply hope you are very contended by your life and the people around you and that you make sure you block us on social media more discreetly!

And well, last but definitely not least...two of the most special people I met in Maastricht, Jo and Yara. Either in Maastricht, Spain, USA or Greece you are definitely the people that stand out from the crowd by far. Jo, the scary, at first sight, most lovable and knowledgeable person. Yara the friendly smiley gal from E-spain that supports Germany in all football matches and votes for the Catalan referendum from wherever she is. What a couple! I don't even know if I can clearly recall all the events we have been through together and well it doesn't matter because our relationship evolved in a much deeper one than just parties. You both have taught me a lot about science, fun, persistence, tolerance, love, sacrifice, respect, and understanding not only through our talks but also through your attitude in life. You are definitely my inspiration and I wish that our friendship survived through distance and time.

While at this era, I could not skip mentioning the contribution of the international dinner group in my integration in the PhD community and also my foodiness. Merhdad, Sanne, Elisa, Joost, Julie, Maarten, Thessely, Giacomo, Paula, Sean and Shuan thank you for all the special, yummy, cultural moments we shared.

Half a year in my second PhD year I moved to Exeter for my one-year internship and boy was that a challenging time; a real emotional seesaw. Luckily I had people next to me to take it one step at a time until everything felt a bit better again. Isabel, Stefania, Therese, Joana, Emma thank you very much for incorporating me in your team that fast. Jonathan, thank you for giving me the chance to see how a fully functional lab group works. It was a true eye-opener! Eilis, thank you for your tolerance as well as for making my brain spin a bit faster either computationally or for sociopolitical issues. Bex, you will always have a very special place in my heart. Your devotion on social matters, gender equality, animal sheltering, as well as your love for cheese, board games and GoT will always fascinate me! Matt, well no matter what we have been through you were one of the most supportive people in Exeter and I definitely wish you all the best for any lifestyle you choose to lead. Nyree and Joe, you are the people encrypted in my fondest memories of Exeter. You showed me that leading an alternative lifestyle is bliss. Our hikes to the Moores, skank-sitting, snake cuddling or even a round of CAH were very cathartic. From the bottom of my heart thank you for everything you did for me.

Upon my return to Maastricht, I was also greeted by some new faces in the department. Roel, Glenn and Perla you reinvigorated the pain group with your fascinating personalities! Paul, thanks you for offering us the amazing Amsterdam experience...and all the extra beers that we always regretted the next morning. Milaine, thank you for comforting my mind with your overworking existence at nights, and for not making that much fun of me that night I was contemplating analysis of that Jameson bottle. Jeroen, our time in the department didn't really align but yes, you could have been among my favourite people too! Sarah, also a huge shout out to you for triumphing through this phase of your life! Shenghua, thank you for opening up my cultural perspectives and introducing me to Chinese cuisine and lifestyle in general! You have created an amazing family, so hurry up with your PhD so that you and Qing can enjoy it to the max! Anne, a big thank you goes to you too for your friendship, and assistance in the fragile times...as well as sharing your ideas about life over nice Greek food! I am sure you are going to rock this next stage of your life!

Gusta, you were also one of my last-semester-highlights of Maastricht. You are witty, funny, positive, optimistic, hard-working, motivated, energetic, and on top of that you like board games and my food! You now possess a special place in my heart and believe it or not at the end of every day I always try to trace the three positive things that happened! For this and your friendship in general I am really grateful! Either we find ourselves picking up kiwis in New Zealand or anywhere else on this globe I am pretty sure we will stay in touch!

And off I went again, this time to Barcelona. Another great dream team there! Aida, Christina, Anaïs, Humberto, Miguel, Edi, Modestos I cannot thank you enough for integrating a non-Spanish speaker like me to your group that fast. The karaoke, the escape rooms, the parties, the cervezas made my short time there so rich! A special thank you to Edi, the single most patient and positive person I know, for all the knowledge transfer and support and of course to Modestos, που εντάξει βρε αδερφέ, όπως και να το κάνουμε το είχαμε το vibe μας.

Just before the epilogue, I would like to thank the people that made the writing process of this thesis enjoyable and gave me this extra gear to keep on going till the last dot of each chapter: Joshua, Alex and Thom.

The last bit of this chapter is dedicated to really special people, that did not let any temporal or spatial variables interfere with our relationship. May you never cease to stain my white-bread lives with supernatural ketchup.

Bethy, our friendship was one of the unpredicatble events of this year. Funny how the southwest, anxiety, red wine, coffee, workaholism, pathological complaining, and love for pressure cleaning devices and doggos bring two seemingly dissimilar people together. You are my gal and you know it! Thanks for standing by me the past year! Jeroen you have such a sweet and kind heart. Our sober or intoxicated chats were absolutely alluring. I am very proud of you and I will always wish for the best for you. You have endured and persevered through so many calamities that you are way stronger than you think and you now you can definately achieve what you set your mind to. And well, I am so thankful that you introduced Louie into my life...this cuddly beast that worked as my stress ball more times than you could have guessed.

Ligia, my Brazilian friend, the person that I know always wishes the best for me. And vice versa...I have the most fond, sweet feelings for you and I am sure that your life will be full of success, good fortune and love. And don't you forget our deal! Hope to see you and Cecília very soon, enjoy the sun and have some brigadeiros! Till then, saudade! Sol, there was something about you since the first time I met you. And I was so right, you turned out to be the Venezuelan version of myself. We saw ourselves in each other, we trusted each other, we built something rather special. I couldn't be more lucky having this connection with you either here in Maastricht or in Barcelona. Thank you for making me feel at home by introducing me to your people and family in Barcelona, sharing your whiskey, your sashimi and, off the record, being so easy to beat at Tekken! I will squeeze in Tim in here as well because his hospitality and kindness significantly helped me get through my first couple of months in Barcelona. To both of you, you are truly awesome, and I wish you all the best for the awe-inspiring future that you are planing!

Roy. You absolutely fascinate me. You are among the smartest, most resourceful, sympathetic, sincere people I have in my life. I should apologise at this point for challenging the borders of your comfort zone, hugging you (too much to your liking), always messing with your recipes, drinking smokey whiskeys from the wrong glass, chugging whiskeys when I am supposed to be tasting them and being a terrible co-player at any PS4 game, but I will not! Because our contrasting yet-peculiarly matching characters made us friends. And a friend like you, reliable, trustworthy, honest, empathic, encouraging and with (black) humor, is one of the best assets I got out of my time in Maastricht. I don't want to dissapoint you but I think you will be extremely successful in your life so we might need to break our promise of collecting oranges in Cuba...bummer!

Marina, Carolin, Shannen...the trips, the food, the wine, the gatherings, the parties, the dances....everything! You spiced up my life. Shannen, you sassy ice queen! Among these things I also want to say how grateful I was for the Christmas dinners we hosted at your place...that was a family moment miles away from home. It was an absolute pleasure to share all these special moments with you. The breakfast visual after our night out in Malta will always put a smile on my face! Cheers! Carolin, apart from a friend, throughout the years you evolved to be my mentor. You are passionate, loving, respectful, fair, polite, kind, optimistic, consientious, responsible; traits that make you a unique human being in this era. Your consious drive and internal motivation in all choices you have made is truly inspirational. Thank you for your friendship! I know we are both terrible with communication, but I truly believe that we will make it work.

Marina, chica, you are the person that branded my time in Maastricht the most. Friend, flatmate, co-foodie, co-fashion lover, co-traveller, co-psychosomatic sufferers, co-'it's ok'ers. Together we created the fondest memories: spontaneous trips to Paris and Hospitalet, or even New York, meetings in London or Barcelona, concert trips to Amsterdam or even London, dancing nights, drinking nights, sofa nights, snapchat videos, deep talking nights, superficial nights, wheneverwherever hashtags, exploring new restaurants, cooking times, no eating times, BBQ days, surprise birthday events, "Felices", regression, development; all that life is about, I experienced it with you. It was absolutely lovely watching you maturing from the naive e-Spanish one to the woman that you are today. We are both aware that things get rough from time to time, but I still do believe that the foundations we set these years will help us persevere and keep the #wheneverwherever promise true throughout the years. You are family.

Freddy, well I could thank you for being a trustworthy proof reader of my thesis and grants, for listening to my weird ideas, for pretending not to value epigenetics just to make me more stubborn, for always giving good medical advice ("Stop taking Ibuprofen!") but instead I will take a different approach. I will instead thank you for surprisingly sharing the same taste in music as me, for lending an ear every time my mood was going downhill, for appreciating good whiskey and sharing it with me as a productivity boost, for always finding time for a coffee in the sun, for helping me start my vinyl collection, for the spark in your eyes and the "yum" moment every time you are tasting my food, for the concerts, for the trips and for all these other things that made you part of the "Felices". It might be only a year of friendship, but I truly cherish how much you helped me throughout. Cheers!

Koen, my oldest Dutch friend. The oldest the relationship the hardest it gets to type down some words, so I will go for one: steadfastness. This is the word that describes our relationship the best. You have been there for there for me in any major event that marked my life the past 5 years. Therefore you would also make the best paranymph since you have experienced all the embarrassing ones as well. You are definitely one of a kind and you will always be!

And here, right before switching to Greek, I will close up by thanking people like James. These people that pop out of nowhere when you need them the most and give you a pat on the back, make you believe in yourself and assist you to stand right back on your feet again. People that sit you down, shut you up, wait

for you to calm down till you are ready to get along with them again. People that challenge you every day with their intellect. People that make your day just by smiling your way. People as rare as a can of dandelion and burdock. People making a leap of faith with you. People worth stopping the world and getting off with them. People that would put the sushi in the fridge and go to the Greek any day. People that when I grow old, sitting in my rocking chair with my slippers on, they will warm my soul and bring a smirk on my face.

Και τώρα ήρθε η ώρα για τις εγχώριες ευχαριστίες.

Το πιο δύσκολο κομμάτι, γιατί δεν είστε άτομα που μπήκατε στην ζωή μου 1-2 χρόνια πριν, αλλά είστε αυτοί που σηματοδοτήσατε και συνεισφέρατε στο να προσδιορίσω την ζωή μου. Εσείς είστε πράγματι οι αφανείς ήρωες αυτής της διατριβής, γιατί ακόμα και αν δεν έχετε ιδέα ποια είναι η δουλειά μου και τι κάνω καθημερινά, είστε πάντα δίπλα μου και με υποστηρίζετε.

Αδελφές Βλάχου, η παρέα σας ήταν ένα ανάλαφρο δροσερό αεράκι στον καύσωνα του αγώνα μου να τελειώσω το διδακτορικό! Σας αγαπώ πολύ! Αλέξανδρε, Ζούμπι μου, πόσα χρόνια, πόσες αναμνήσεις, πόσες περιπέτειες και πόσα ακόμα σκαμπανεβάσματα. Η συμπαράσταση σου, η στήριξη σου και η φιλία σου γενικότερα είναι πολύτιμες για εμένα! Σ'ευχαριστώ πάρα πολύ!

Τσαλαβρέττα, μπορεί να έχουμε 99 προβλήματα, αλλά ένα ακόμα τεταρτόξαδερφο δεν είναι ένα από αυτά! Είμαι πάρα πολύ περήφανη για ό,τι έχεις καταφέρει στη ζωή σου και είμαι σίγουρη ότι όλα θα κυλήσουν ομαλά από εδώ και πέρα. Ξέρεις πόσο εκτιμώ την πίστη σου σε εμένα, την συμπαράσταση σου, το ότι δεν μ'αφήνεις ποτέ να κερδίσω στο φεύγα και την παρέα σου στις εύκολες, δύσκολες και κρύες στιγμές! Ευχαριστώ από τα βάθη της καρδιάς μου.

Ίρις, όπως είχες πει και εσύ, είμαστε η πιο φωτεινή υπενθύμιση του τι έχουμε καταφέρει και που μπορούμε να φτάσουμε! Νομίζω ότι είμαστε σε πολύ καλό δρόμο! Όσο περνάνε τα χρόνια θα κοιτάμε πίσω και θα χασκογελάμε ή θα μας βρίζουμε για τις επιλογές που έχουμε κάνει, αλλά όπως και να έχει θα έχουμε η μία την άλλη να πορευόμαστε σταδιακά προς τους στόχους μας ή να τους επαναπροσδιορίζουμε. We have 24 years of friendship to prove that!

Μάριε και Σοφία, εσείς χρειάζεστε ένα ξεχωριστό κεφάλαιο. Όπου και να βρισκόσαστε με βοηθάτε να ξεφεύγω από την πραγματικότητα και να μπαίνω σε αυτό το παράλληλο σύμπαν που έχετε δημιουργήσει. Με κάνετε να νιώθω σαν στο σπίτι μου και για την ακρίβεια, με κάνετε να έχω ένα σπίτι όπου και να βρίσκεστε.

Ευχαριστώ πολύ για το έναυσμα που μου δίνετε πάντα να κάνω το επόμενο βήμα! Είστε ξεχωριστοί άνθρωποι, και ελπίζω να παραμείνετε μέρος της ζωής μου ανεξαρτήτως της απόστασης.

Τέλος, θα ήθελα να ευχαριστήσω την οικογένεια μου. Αρχικά εσένα, Γιώργο, που είσαι πάντα το πιο λαμπρό παράδειγμα του τι μπορώ να καταφέρω στη ζωή μου! Σ'ευχαριστώ πολύ που είσαι πηγή έμπνευσης μέσα από τις επιλογές σου όλα αυτά τα χρόνια!

Δήμητρα, η υπέρμετρη πίστη σου σε εμένα μου δίνει ώθηση να συνεχίζω κάθε φορά που αντιμετωπίζω δυσκολίες. Σε ευχαριστώ πολύ!

«I would like to thank my wonderful majestic sister for her impeccable wisdom and her limitless sense of humour.»

Τάνια, είσαι η πρώτη μου ευχή που βγήκε αληθινή. Έκτοτε είσαι ό,τι καλύτερο συνέβη στη ζωή μου. Μου δίνεις δύναμη κάθε μέρα και με κάνεις να είμαι η καλύτερη βερσιόν του εαυτού μου για εσένα (ξέρω ξέρω, τα τελευταία χρόνια δεν φαίνεται τόσο αλλά πίστεψε με προσπαθώ). Όσο και να μην το πιστεύεις, σε θαυμάζω για το πόσο έχεις ωριμάσει και για την προσωπικότητα που έχεις αναπτύξει. Επίσης ζηλεύω λίγο που όλο το χιούμορ το πήρες εσύ! Δεν ξέρω πως θα είχαν κυλήσει τα τελευταία χρόνια αν δεν είχα εσένα δίπλα μου. Είσαι ο άνθρωπος που μπορώ να μοιραστώ τα πάντα, το χαμόγελο μου, η καλύτερη μου φίλη, η αδερφή μου.

Μαμά και μπαμπά, σας χρωστάω τα πάντα. Είμαι εδώ που βρίσκομαι αυτή τη στιγμή επειδή μου δώσατε όλα τα εφόδια για να σταθώ στα πόδια μου. Με αναθρέψατε με τον καλύτερο τρόπο και με κάνατε τον άνθρωπο που είμαι σήμερα. Η αγάπη σας και η υποστήριξη σας όλα αυτά τα χρόνια μου έχει δώσει φτερά. Η ενθάρρυνση και παρότρυνση σας με έχει κάνει να συνεχίζω να παλεύω ακόμα και όταν αμφιβάλλω για τον εαυτό μου και τις ικανότητες μου. Το βλέμμα σας και τα λόγια σας κάθε φορά που καταφέρνω κάτι δίνει νόημα στη ζωή μου και με κάνει να πεισμώνω ακόμα παραπάνω για να μην σας απογοητεύσω ποτέ. Σας αγαπώ πολύ και σας ευχαριστώ για τα πάντα!