

## EXPERT REVIEW

## Opening up the DNA methylome of dementia

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Dementia is a complex clinical condition characterized by several cognitive impairments that interfere with patient independence in executing everyday tasks. Various neurodegenerative disorders have dementia in common among their clinical manifestations. In addition, these diseases, such as Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies and frontotemporal dementia, share molecular alterations at the neuropathological level. In recent years, the field of neuroepigenetics has expanded massively and it is now clear that epigenetic processes, such as DNA methylation, are mechanisms involved in both normal and pathological brain function. Despite the persistent methodological and conceptual caveats, it has been reported that several genes fundamental to the development of neurodegenerative disorders are deregulated by aberrant methylation patterns of their promoters, and even common epigenetic signatures for some dementia-associated pathologies have been identified. Therefore, understanding the epigenetic mechanisms that are altered in dementia, especially those associated with the initial phases, will allow us not only to understand the etiopathology of dementia and its progression but also to design effective therapies to reduce this global public health problem. This review provides an in-depth summary of our current knowledge about DNA methylation in dementia, focusing exclusively on the analyses performed in human brain.

*Molecular Psychiatry* (2017) **22**, 485–496; doi:10.1038/mp.2016.242; published online 3 January 2017

## INTRODUCTION

Current demographic trends are leading to a world population comprising a higher proportion of older people. By 2050, for the first time in history, there will be fewer young people than older people.<sup>1</sup> This not only poses a major environmental problem<sup>2</sup> but will also give rise to an incalculable worldwide socioeconomic burden because of age-related disorders. At present, more than 48 million people worldwide live with dementia and it is estimated that, owing to the lack of preventative therapeutics, there will be more than 130 million patients by 2050.<sup>3</sup> The estimated global cost of dementia is US\$818 billion for 2016,<sup>4</sup> although this figure does not take into account the incalculable social and health costs to relatives and other caregivers.

Dementia is not defined as a specific disease but rather as a collection of cognitive impairments caused by a plethora of disorders. Typically, dementia symptoms include impaired cognitive functions such as loss of memory, language and constructional skills, which are often accompanied by behavioral alterations such as delusions or agitation. Clinical diagnosis of dementia requires the manifestation of two core features characterized by a loss of intellectual ability of sufficient severity to interfere with the patient's independence.<sup>5</sup> Some of the diseases that feature dementia are Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD; Pick's disease).<sup>6</sup>

Recent investigations have indicated that neuroepigenetic mechanisms are key players in dementia-associated disorders, supporting the hypothesis that the accumulation of epigenetic alterations over the lifespan could be a mechanism underlying these disorders (the 'Latent Early-life Associated Regulation'

(LEARn) model).<sup>7,8</sup> Neuroepigenetic processes are complex mechanisms that regulate the transcriptome of brain cells in serving several functions, such as learning and memory,<sup>9</sup> and in the aging process<sup>10–12</sup> and pathological states.<sup>13</sup> Epigenetic mechanisms are characterized by their ability to alter transcription, by changing the chromatin structure without modifying the DNA sequence.<sup>14</sup> The best understood type of epigenetic modification is DNA methylation,<sup>15</sup> which occurs by covalently adding a methyl group from S-adenosyl methionine to the 5' position of cytosines (5 mC) linked by a phosphodiesterase bond to guanines (CpG dinucleotides). Promoter regions of coding genes (~70% of human genome promoters) are frequently highly enriched in CpGs, forming the CpG islands.<sup>16</sup> DNA methylation is generally considered to be a repressive mark<sup>17,18</sup> that principally attracts other proteins associated with gene silencing.<sup>19</sup> The addition of methyl groups to the DNA is catalyzed by a set of enzymes called DNA methyltransferases (DNMTs), a group formed by DNMT1, DNMT2 and DNMT3 proteins.<sup>20</sup> All of these proteins are strongly expressed in neurons,<sup>21</sup> although their function is quite different. While DNMT1 is responsible for maintaining the methylation pattern after cell division,<sup>22</sup> DNMT3a and DNMT3b are responsible for the *de novo* methylation.<sup>23,24</sup> DNA methylation is a reversible process owing to the function of the TET proteins that, through the oxidation of the methyl group, lead the conversion of 5 mC in 5-hydroxymethylcytosines (5 hmC),<sup>25</sup> which after successive conversions to 5-formylcytosine and 5-carboxycytosine definitively erase the epigenetic mark. Brain cells are particularly highly enriched in 5 hmC,<sup>26</sup> and it has been postulated that it is not merely an intermediate state in the demethylation process but, in fact, a key epigenetic mark in neurological disorders.<sup>27,28</sup>

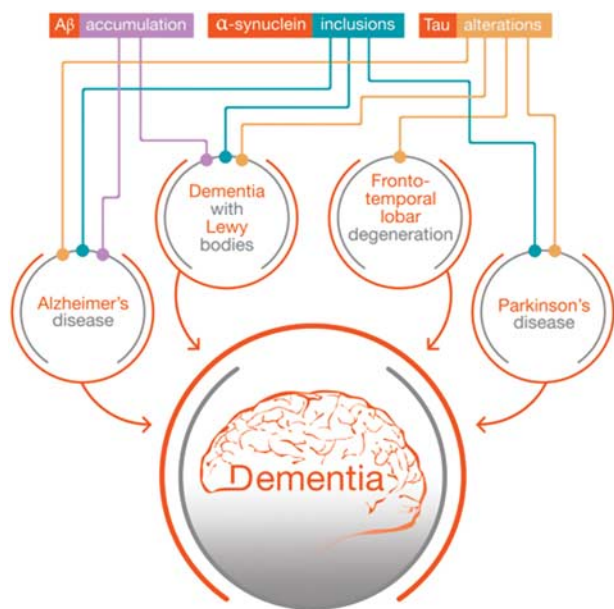
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Received 27 May 2016; revised 2 November 2016; accepted 14 November 2016; published online 3 January 2017

Alterations in the DNA methylation pattern have been seen in other cellular genomes, such as that of the mitochondria. The mitochondrial genome (mtDNA) is small (16.6 kb) and comprises 37 genes. Although it does not contain CpG islands it is highly regulated by DNA methylation processes.<sup>29,30</sup> The existence of both 5 mC and 5 hmC in the mtDNA<sup>30</sup> and the presence of mitochondrial DNMT and TET proteins have been confirmed.<sup>30,31</sup> Several laboratories have recently turned their attention to the role of the (hydroxy)methylome of mtDNA and its function regulating gene expression.<sup>32</sup> Little is known about brain mtDNA methylation but we do know that increased 5 hmC levels are associated with aging in mice.<sup>33</sup>

Several disorders with an apparently diverse spectrum of symptoms feature dementia. Surprisingly, those brain alterations exhibit similar molecular alterations, in spite of the observed neuroanatomical differences in the affected areas among these disorders. For example, amyloid plaques, a hallmark of AD, are also formed in DLB, and Tau alterations are common in AD, PD, DLB and FTD.<sup>34–39</sup> These disorders even have similarities at the genetic level, where it has been shown that DLB is equally correlated with AD and PD,<sup>40</sup> implying that similar mechanisms are involved (Figure 1). However, it is also possible that different set of alterations or perturbation in brain cells lead to similar response at the genetic level. Our laboratory has recently demonstrated that several dementia-related neurodegenerative disorders (AD, PD, DLB and Down syndrome) share a common set of genes undergoing aberrant DNA methylation shifts.<sup>41</sup> These genes, which are involved in functions such as cortex development and general gene regulation, highlight the importance of epigenetic alterations in disorders associated with dementia, despite the limiting number of articles performing cross-disorder profiles. In this review, we will focus on the role of DNA (hydroxy)methylation in dementia-related disorders by summarizing the current knowledge of the role of this particular epigenetic modification in human neurodegeneration. Overall, relevant gene targets for



**Figure 1.** Schematic representation of the known connections between Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies and frontotemporal dementia. These disorders not only feature dementia as one of their core clinical manifestations but also share neuropathological alterations such as amyloid- $\beta$  accumulation, Tau alterations (hyperphosphorylation or loss of function) and  $\alpha$ -synuclein aggregation (Lewy bodies).

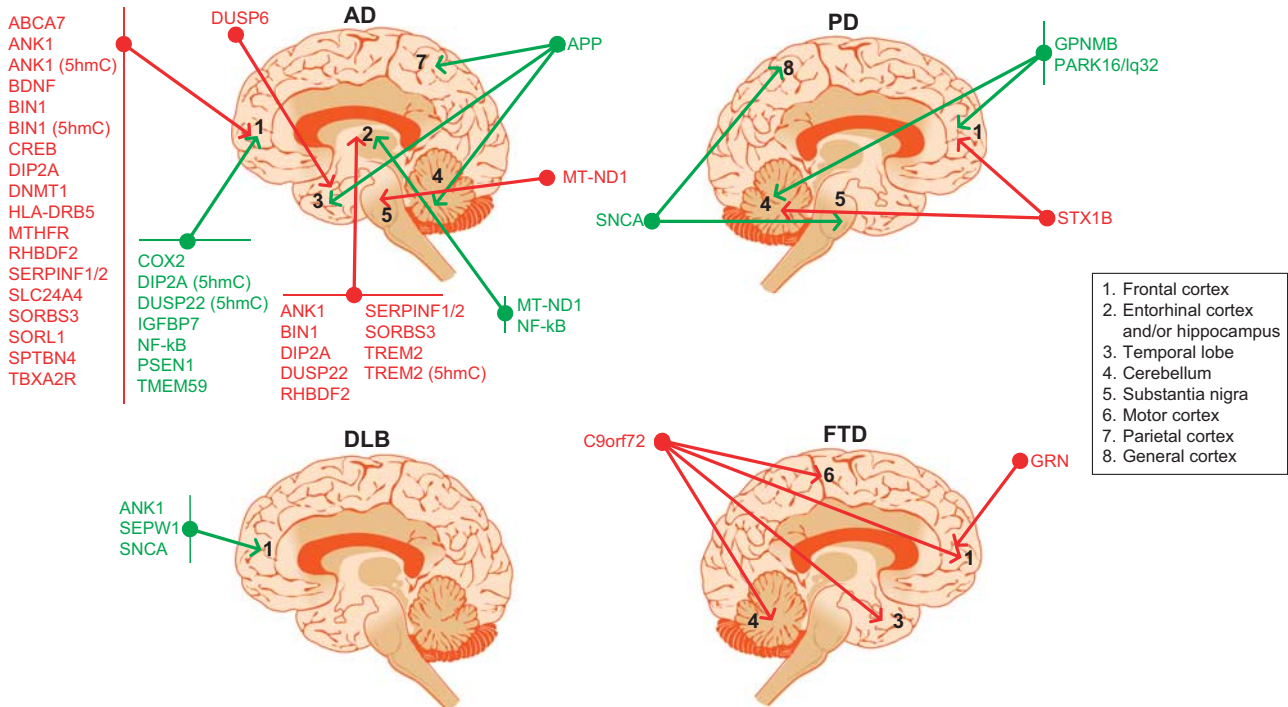
altered DNA methylation in dementia are illustrated in Figure 2, whereas Figure 3 shows the intracellular location of the encoded proteins. The next sections will analyze in detail these epigenetic changes across the broad spectrum of clinical entities associated with dementia.

## ALZHEIMER'S DISEASE

AD is the leading form of dementia worldwide. It is an age-related neurodegenerative disorder that severely impairs memory and other cognitive functions. Although clinical manifestations typically occur in patients over 65 years of age, AD develops over decades, and it is believed that the first pathogenic processes start several decades before typical symptoms manifest themselves.<sup>42,43</sup> At the molecular level, AD is characterized by the appearance of two neuropathological hallmarks: senile plaques, comprising insoluble aggregates of amyloid  $\beta$ , and neurofibrillary tangles, which are comprised of abnormally hyperphosphorylated Tau.<sup>44,45</sup> Although the scientific community has amassed considerable knowledge of the molecular alterations and temporal progression of the disease, the events driving the origin of the disorder are unknown. Genetic and non-genetic factors contribute to the etiopathology of AD. To date, almost 300 mutations have been associated with familial AD, especially those in the *APP*, *PSEN1* and *PSEN2* genes.<sup>46</sup> However, only ~5% of all AD cases are mainly caused by genetic factors, which draws attention to the need to decipher the non-genetic causative factors of late-onset AD, which is most likely triggered by environmental stimuli that participate in the appearance of AD.

In the past few years, researchers have begun to realize that epigenetic mechanisms are key players in the development/course of AD. Age-associated alterations at the levels of 5 mC and 5 hmC and other epigenetic marks<sup>47,48</sup> have been associated with the progression of AD.<sup>49,50</sup> Several AD-associated genes have been analyzed at the epigenetic level. The *APP* gene has been described, by using targeted approaches, as hypomethylated in multiple CpG sites on its promoter,<sup>51–53</sup> a concomitant finding with the upregulated levels of APP found in AD brains. These studies used a small cohort, and the findings could not be confirmed in a larger cohort.<sup>54</sup> These conflicting results highlight the necessity of performing better and more in-depth studies on the regulation of the *APP* gene by epigenetic mechanisms. Other *APP*-related genes, such as *APOE*, *Nicastrin* and *BACE1* (which is hypomethylated in peripheral blood mononuclear cells of AD patients<sup>55</sup>), were found to be unaltered in AD brain samples, whereas *PSEN1* was hypomethylated and other genes, such as *MTHFR* and *DNMT1*, were hypermethylated.<sup>56</sup> Analyses involving matrix-assisted laser desorption ionization-time of flight mass spectrometry are difficult to interpret, especially because of the small sample sizes used and the high interindividual variance observed, particularly in the *PSEN1* and *APOE* genes. Interestingly, *SORL1* and *ABCA7*, which are genes related to A $\beta$  production, were found to be hypermethylated in AD brains.<sup>57</sup> Methylation levels of other genes associated with the amyloid machinery, such as *DIP2A*, *SERPINF1*, *SERPINF2*<sup>49</sup> and *TMEM59*,<sup>58</sup> are also altered, although the specific meaning of such findings needs to be rigorously addressed. Importantly, the DNA methylation alterations found in De Jager *et al.*<sup>49</sup> correlated with the burden of amyloid plaques identify on the brain tissue, thus associating their finding to the neuropathological state of the subjects.

Other targeted analyses of key molecular players of AD have shown epigenetic alterations in the Tau cascade. Our team demonstrated that *DUSP22*, a phosphatase-targeting abnormal Tau, presents a hypermethylated promoter in AD that is associated with the lower levels of the protein described in the pathology.<sup>59</sup> The levels of methylation of *DUSP22* correlated positively with Braak stage of the AD patient. Accordingly, the promoter of the *DUSP6* gene, another phosphatase that regulates Tau, was found



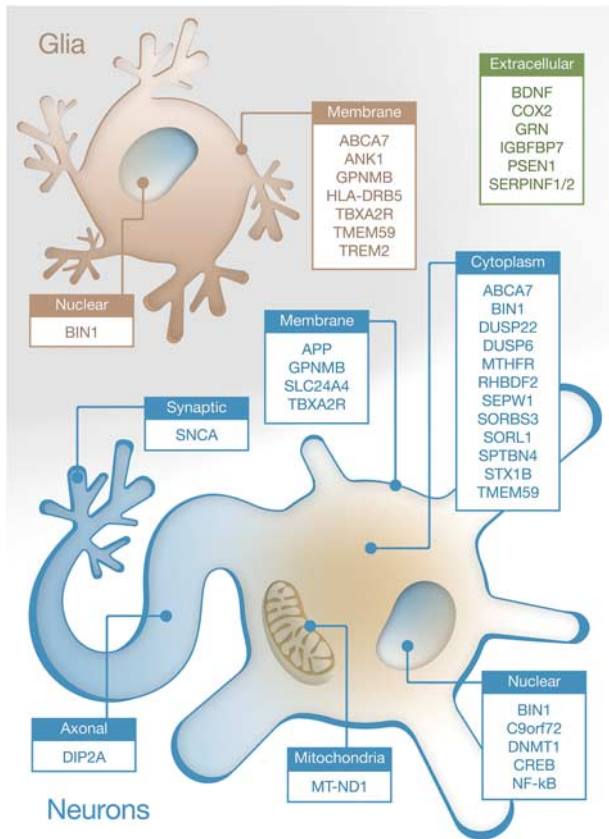
**Figure 2.** Illustration to summarize the major DNA (hydroxy)methylation changes described to date in Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD) among different brain regions. Red, hypermethylated genes in the disorder; green, hypomethylated genes in the disorder.

to be hypermethylated in AD brain<sup>60</sup> using a genome-wide approach. Although *TAU* promoter is not regulated via DNA methylation,<sup>54</sup> the data obtained indicate that the molecular cascade associated with Tau hyperphosphorylation or dephosphorylation may be deregulated epigenetically in AD. In another genome-wide study, other molecules associated with Tau alterations, such as *BIN1*,<sup>61</sup> were found to be aberrantly hypermethylated in AD,<sup>49,57</sup> a change correlated with the amyloid plaques burden. Other molecules associated with neuronal function and firing potentials, such as *SPTBN4* (spectrin beta 4), are down-regulated in human AD brains, a finding that correlates with the hypermethylation status of its promoter.<sup>62</sup> *SPTBN4* not only participates in the formation of the axon initial segment but also anchors CaMKII to it. CaMKII mislocalization, due to a loss of *SPTBN4*, is a plausible mechanism for Tau hyperphosphorylation.<sup>63</sup> Overall, those sets of data indicate that the two major molecular hallmarks in AD could be regulated via DNA methylation in the early and late phases of the disorder.

Several studies have highlighted the importance of epigenetic processes in the gene expression deregulation occurring in AD, in addition to identifying the AD hallmarks. By analyzing the frontal cortex of human post-mortem samples of AD patients, two independent groups found AD-associated hypermethylation of the promoter of *SORBS3*, a protein involved in synaptic function.<sup>62,64</sup> Although this effect has been associated with normal aging, this hypermethylation seems to occur faster in AD. Recently, epigenome-wide association studies have identified *ANK1* (ankyrin1) as a potential biomarker of AD, as its methylation level is strongly correlated with early stages of the disease and strongly associated with Braak score of the patient. In AD, it is downregulated via hypermethylation of its promoter.<sup>41,49,65</sup> Little is known about *ANK1* function, but it seems to be related to microglia activation and cell motility. Other molecules associated with the immune response show aberrant methylation states. For example, *RHBDF2* (related to TNF $\alpha$  function) and *HLA-DRB5* are

hypermethylated, and nuclear factor- $\kappa$ B (*NF- $\kappa$ B*) and *COX2* (cyclooxygenase-2), which are proinflammatory and associated with inflammatory events in AD, are hypomethylated.<sup>41,49,57,66,67</sup> Mutations of the gene coding for *TREM2* (triggering receptor expressed in myeloid cells 2) have been associated with an increased risk of suffering AD.<sup>68,69</sup> *TREM2* is expressed in microglia and is associated with microglia activation, phagocytosis and the immune response.<sup>70</sup> *TREM2* mRNA levels are upregulated in AD hippocampus, its mRNA levels being positively correlated with the AD Braak stages. Interestingly, the increase in mRNA levels are accompanied by a Braak-associated hypermethylation of the *TREM2* promoter.<sup>71</sup> These results are not those expected from the methylation of promoters, which are normally associated with gene repression, and further research is needed to understand these effects. Overall, synaptic degeneration and immune changes are some of the best known alterations in the pathophysiology of AD,<sup>72,73</sup> and there are evidences that suggests that those are influenced by epigenomic modifications.<sup>74</sup>

Other molecules have been identified as being epigenetically deregulated in AD. Demyelination processes have been associated with aging but are accelerated in AD, and a recent study has shown that transcriptional alterations in myelination-associated proteins, such as *WIF1* and *RARRES3*, may be mediated via DNA methylation changes.<sup>75</sup> *SLC2A4*, which is thought to be involved in neural development, is also hypermethylated in AD brains.<sup>57</sup> Glucose metabolism in brain is altered in this pathological state<sup>76</sup> via deregulation of the brain insulin signaling cascade.<sup>77,78</sup> Regarding this pathway, *IGFBP7* (insulin-like growth factor binding protein 7) is upregulated in the brains of AD patients probably because of the hypomethylated state of its promoter.<sup>79</sup> This protein binds to insulin growth factors and attenuates its protective effects.<sup>80</sup> Rao *et al.*<sup>66</sup> demonstrated that the reduction in brain-derived neurotrophic factor previously observed in AD<sup>81</sup> was probably due to hypermethylation of its promoter. This epigenetic effect observed in the prefrontal cortex of AD patients



**Figure 3.** Representation of the known intracellular location of proteins encoded by genes undergoing aberrant DNA methylation in dementia. Nuclear, cytosolic or membrane-associated genes both in neurons (blue) or glia (principally astrocytes and microglia; in pale brown) are illustrated, together with secreted (extracellular) proteins (green).

was accompanied by a similar one at the promoter of the cAMP response element-binding protein (*CREB*).<sup>66</sup> Both brain-derived neurotrophic factor and CREB are critical proteins for neuronal survival.<sup>82,83</sup> Other authors have also described alterations in genes related to CREB signaling, such as hypermethylation of *TBXA2R* (thromboxane A2 receptor) gene promoter,<sup>62</sup> a G-protein receptor regulating CREB, thereby highlighting the importance of CREB signaling in AD.<sup>84</sup>

Besides studying the 5 mC levels in AD, researchers have begun to analyze the role of 5 hmC in health and disease. The central nervous system is especially enriched in 5 hmC relative to other body tissues,<sup>26</sup> and exhibits a specific pattern across the different brain areas.<sup>85</sup> 5 hmC is considered to be a key epigenetic mark in brain development and neurological disorders,<sup>27,28</sup> leading researchers to postulate that age-associated neuronal 5 mC/5hmC changes may be involved in the early and late phases of AD. There are lower 5 hmC levels in several brain regions of late stages of AD patients, such as the neocortex,<sup>86</sup> hippocampus,<sup>87</sup> entorhinal cortex and cerebellum.<sup>88</sup> A genome-wide study has recently found several genes to be associated with neuronal morphology and synaptic function with aberrant 5 hmC levels in the prefrontal cortex of AD patients. Bernstein *et al.*<sup>89</sup> found differential 5 hmC levels of *ANK1*, *DIP2A*, *BIN1* and *DUSP22* genes among those genes described as being epigenetically altered in AD.<sup>41,49,59,65</sup> *TREM2*<sup>69</sup> also presents alterations in 5 hmC levels in AD brains, insofar as there is a correlated increase in 5 hmC levels in exon 2 and *TREM2* mRNA levels.<sup>71</sup> However, there are several controversies regarding

this epigenetic mark, reflecting the need for further studies to determine the exact role of 5hmC in AD. For example, there are raised levels of 5 hmC in the hippocampus<sup>90</sup> and temporal gyrus<sup>50</sup> of AD patients. These discrepancies could have arisen as a result of studying different brain populations (i.e. entorhinal cortex vs hippocampus vs temporal gyrus) but also for technical reasons (i.e. immunohistochemistry vs western blotting or dot blot).

Owing to the fact that mitochondrial alterations have been found in AD,<sup>91–93</sup> scientific community have started to analyze the role of the (hydroxy)methylome of mtDNA in brain (mal) function.<sup>32</sup> In the context of AD, Blanch *et al.*<sup>94</sup> found a global reduction of 5 mC in mtDNA in mid–late stages of AD (Braak III/IV). Analysis of specific loci showed that the levels of 5 mC in *MT-ND1*, a protein of the complex I of the respiratory chain, were lower in the entorhinal cortex of AD patients compared with that in age-matched controls. However, no changes were noted in 5 hmC of the mtDNA of AD samples despite that it was described previously as a reduction in 5 hmC levels in aging-associated mtDNA.<sup>33</sup> The discrepancy between the Blanch and Dzitoyeva studies could be due to differences between species (humans vs mice, respectively) and the methods used (hydroxyMeDIP assay vs ELISA).

Overall, it is clear that the methylation of DNA is critical to epigenetic processes associated not only with normal brain function and aging but also with AD. Although we still lack information regarding the specific patterns of methylation in specific neuronal populations of key brain nuclei, transcriptomic alterations in AD via epigenetic deregulations could drive the induction of alterations in molecular cascades that will eventually end up with the induction or acceleration of the appearance of AD hallmarks and, consequently dementia and neurodegeneration. The ongoing search for more epigenetic modifications<sup>95</sup> will require the development of new technologies to improve our knowledge of the specific role of DNA modification in brain function. Data from a combination of better analyses of human AD samples that take into account the heterogeneity of brain tissue by studying the neuroepigenome via strategies such as single-cell epigenomics,<sup>96</sup> and base-resolution whole-genome studies of large cohorts of human samples,<sup>97,98</sup> are sure to be of value for designing early therapeutic interventions aimed at preventing the initial alterations of and, ultimately, AD itself.

### PARKINSON'S DISEASE

PD is a relatively frequent form of neurodegeneration with a prevalence of ~2% worldwide in the over 70 years of age,<sup>99</sup> affecting more than four million people<sup>100</sup> and with an estimated incidence of 1% in people over 60 years of age.<sup>101</sup> PD is characterized by a plethora of symptoms progressing from non-motor changes (olfactory impairments, sleep alterations, constipation and depression)<sup>102</sup> to motor alterations (tremor, bradykinesia, rigidity).<sup>103</sup> Although the diagnosis of PD is based on the appearance of the motor symptoms, current knowledge indicates that the psychiatric manifestations of the disorder are the first to appear. At the pathophysiological level, PD is characterized by an accumulation of  $\alpha$ -synuclein in diverse brain areas, accompanied by a loss of dopaminergic neurons in the substantia nigra and catecholaminergic neurons in the locus coeruleus.<sup>15,102</sup> The brains of PD patients often present Lewy bodies, which are formed by the accumulation of  $\alpha$ -synuclein, and by parkin and ubiquitin aggregates.<sup>104</sup> Although its cause remains unknown, it has been hypothesized that the disorder is influenced by the interaction of genetic and non-genetic factors, both contributing to a progressive neuronal loss in different brain areas. Monogenic cases of PD, directly affecting ~5–10% of all cases, have been described for mutations in the *ATP13A2*, *DJ1*, *LRRK2*, *PARK2*, *PINK1* and *UCHL-1* genes.<sup>105,106</sup> In addition to the autosomal dominant mutations, several risk loci have also been identified.<sup>107</sup> However, the majority of PD cases are sporadic, their origin being unknown.

Parkinson's disease dementia (PDD) is a late symptom affecting up to 80% of patients with PD.<sup>108</sup> Clinical manifestations of PDD not only include the classic memory impairments but also a myriad of phenotypes, such as alterations of the executive function, deficits in recognition memory, attention processes and visual perception, often accompanied by visual hallucinations and cognitive fluctuations.<sup>109</sup> However, the mechanisms underlying PDD are still not understood. Recently published data on the epigenetics of PD have suggested the involvement of this regulatory mechanism in the etiopathology of the disorder, although the exact involvement of DNA methylation in the initiation or progression of PD is still unclear.

Most of the analyses of the role of DNA methylation in PD/PDD have confined themselves to investigating the regulation of genes causing PD. The promoter of *SNCA*, the gene responsible for the expression of the presynaptic  $\alpha$ -synuclein, is hypomethylated at intron 1 in several brain areas such as the substantia nigra and cortex of PD patients.<sup>110–112</sup> This epigenetic regulation of *SNCA* could explain the higher level of expression of  $\alpha$ -synuclein found in PD brains and other neurodegenerative disorders that feature dementia.<sup>113</sup> Interestingly,  $\alpha$ -synuclein is capable of sequestering DNMT1 from the nucleus, a factor that could explain the global hypomethylation observed in human PD brains.<sup>112</sup> Nonetheless, more in-depth studies of *SNCA* epigenomic regulation are required, as other methylomic studies of PD have not been able to detect differences in *SNCA* intron 1 methylation levels.<sup>41,114</sup> Besides *SNCA*, no other genes directly related to PD have been identified as being regulated via methylation of their promoters. Several studies using genome-wide approaches have failed to identify differences in DNA methylation of *LRKK2*, *Parkin*, *UCHL-1*, *MAPT* and *ATP12A2* promoters.<sup>41,115,116</sup> However, genes such as *PARK16/lq32*, *GPNMB* and *STX1B*, associated with an increased risk of PD, showed altered methylation levels in PD brains.<sup>115</sup> Other analyses taking genome-wide approaches identified alterations of several genes at the methylation level.<sup>41,117</sup> Interestingly, methylation levels of the *NPAS2* gene, a clock gene, were lower in PD brains.<sup>118</sup> Sleep disturbances are a core symptom of PD<sup>119</sup> and *NPAS2* is involved in sleep regulation.<sup>120,121</sup>

Little is known about 5 hmC changes in the brain of PD patients; the only data available have come from the 6-hydroxydopamine-induced rat model of PD. Using that model, Zhang *et al.*<sup>122</sup> were unable to find any difference in the levels of 5 hmC in rat brains.<sup>122</sup> Therefore, the potential involvement of 5 hmC in the causes or consequences of PD remains to be determined. Overall, little is known about aberrant DNA (hydroxy)methylation patterns in PD/PDD but the information obtained highlights the need for further research on the neuroepigenomics of PD, as the data collected so far indicate that epigenetic processes may mediate some of the molecular alterations observed in this pathology.

## DEMENTIA WITH LEWY BODIES

Besides PD, there are other forms of dementia induced by the formation of Lewy bodies (abnormal aggregates of proteins, principally  $\alpha$ -synuclein). DLB is a common form of dementia that differs from PD by the absence of parkinsonism and the progression of the pathology. PD and DLB are classified together as Lewy body dementias and are considered as a continuum rather than as distinct diseases, DLB being an early manifestation of dementia in PD patients.<sup>108</sup> DLB is the second most common form of age-related dementia, accounting for an estimated 16–24% of all dementias.<sup>123</sup> Although there is still no clear information about its neuropathological changes, DLB shares several pathological marks with AD, PD and PDD: the presence of altered A $\beta$  levels both in DLB and AD has been described, and several mutations associated with AD or PD have also been related to DLB, including mutations in *APP*, *PSEN1/2*, *SNCA* and *LRKK2*.<sup>40,124</sup> The clinical diagnosis of DLB focuses on the cognitive impairments

associated with confusion and attentional deficits, especially those related to visuospatial function.<sup>125</sup> These symptoms are often accompanied by psychiatric alterations such as hallucinations, apathy and anxiety.<sup>126,127</sup>

Although the molecular driving events responsible for the development of DLB are still unknown, several authors have identified gene networks that are epigenetically deregulated in DLB. Humphries *et al.*<sup>75</sup> demonstrated that DLB brain tissue features DNA methylation alterations in genes related to synaptic function and behavioral response. This is not surprising since cognitive decline is one of the major alterations occurring in DLB.<sup>9,128</sup> Although A $\beta$  overproduction is common in DLB, one study failed to describe DNA methylation changes in human brains of DLB patients in the *APP* gene<sup>53</sup> or other AD-related genes.<sup>54</sup> Sleep disturbances are also common in DLB, especially rapid eye movement sleep behavior disorder.<sup>129</sup> It is of note that DNA methylation deregulation of the clock genes *PER1* and *CRY1*, which help regulate the circadian cycle and sleep,<sup>130</sup> have been reported in DLB blood samples,<sup>131</sup> although it will be necessary to analyze the effects on the central circadian clock. Desplats *et al.*<sup>112</sup> described a global hypomethylation state in DLB post-mortem brain samples that probably arises from the reduction in Dnmt1 nuclear levels. The same authors not only described the general hypomethylation state but also reported that the promoter of the *SNCA* and *SEPWI* genes, which are associated with PD,<sup>110,111,132</sup> was also hypomethylated in DLB. Very recently, our team reported that the DNA methylation level of the promoter of *ANK1*<sup>65</sup> was altered in DLB, along with genes related to several pathways, such as MAPK, ErbB, neurotrophin, mTOR, p53 signaling and regulation of the actin cytoskeleton.<sup>41</sup>

In conclusion, further analysis of the epigenetic alterations in DLB is required. Owing to the molecular alterations common to that disorder, PDD and AD, the identification of epigenetically deregulated genes common to all these disorders<sup>41</sup> will provide invaluable knowledge of the early changes in DLB. A better understanding of aberrant DNA methylation, and an analysis of 5 hmC specific to DLB are necessary to enable the better stratification and diagnosis<sup>108</sup> of patients, and to accelerate the development of definitive therapeutic approaches.

## FRONTOTEMPORAL DEMENTIA

FTD is the second most common form of neurodegenerative disease and the third most common form of dementia, after AD and Lewy body dementias (PDD and DLB). It has an estimated prevalence of 3–26% worldwide in people older than 65 years of age.<sup>133</sup> FTD is a heterogeneous disorder comprising various subtypes, including behavioral-variant frontotemporal dementia (the most common form of the disease, which accounts for ~70% of the cases of FTD) and the primary progressive aphasia, including the semantic-variant primary progressive aphasia and the non-fluent variant primary progressive aphasia.<sup>134,135</sup> Clinical features of FTD vary depending on the subtype of the disorder, although symptoms may converge. In general, FTD is characterized by the presence of cognitive and executive impairments, spatial skills and memory deficits, decline in social conduct and deficits of speech.<sup>136</sup> Around 10% of FTD patients develop motor neuron disease and early parkinsonisms.<sup>133</sup> All these symptoms are associated with a bilateral frontal and anterior temporal lobe degeneration (frontotemporal lobar degeneration; FTLD), and degeneration of the striatum. At the histological level, FTD is characterized by neuronal loss (especially of large cortical nerve cells) and gliosis accompanied by microvacuolar changes. Other frequently observed alterations are the presence of argyrophilic neuronal inclusions, known as Pick bodies, which are positive for Tau and ubiquitin proteins, and swollen cells.<sup>133,136,137</sup>

Approximately 40% of FTD patients report a history of dementia in the family.<sup>138</sup> The most frequent mutations are found in the

microtubule-associated protein Tau<sup>139</sup> (*MAPT*) and in the growth factor progranulin (*GRN*) gene<sup>140,141</sup> (which accounts for > 50% of the inherited cases) and on the gene of the chromosome 9 open reading frame 72 (ref. 142) (*C9orf72*; ~25% of the cases). Other rare mutations appear in genes such as *TARDBP*, *FUS*, *CHMP2B*, *TREM2* and *VCP*.<sup>133,143</sup> However, most FTD cases are sporadic, with molecular alterations of unknown origin.

Although a genome-wide analysis of the methylomic alterations in FTD has not been undertaken, some groups have investigated the role of DNA methylation in the regulation of specific genes associated with the risk of FTD. Two of the most prevalent mutated genes, *GRN* and *C9orf72*, have been found in aberrant methylomic state in FTD. The *GRN* gene is hypermethylated in FTD brains relative to age-matched controls, but notably, also in comparison with AD and PD samples.<sup>144</sup> The increased

methylation was correlated with lower levels of GRN protein in the frontal cortex of FTD patients. Another of the genes associated with FTD, *C9orf72*, is epigenetically altered in patients' brains. Expansion of the G<sub>4</sub>C<sub>2</sub> repeat located in the proximal region of *C9orf72* is a pathological common cause of FTD.<sup>145</sup> Aberrant hypermethylation has been described in the G<sub>4</sub>C<sub>2</sub> repeat,<sup>146</sup> and in the CpG island flanking the region of *C9orf72*.<sup>147-150</sup> The size of the G<sub>4</sub>C<sub>2</sub> repeat is positively correlated with the level of hypermethylation.<sup>151</sup> This hypermethylation correlates positively with a reduced neuronal loss, thus pointing out that the downregulation of the genes could have a protective effect in the hippocampus and frontal cortex of FTD patients' brains.<sup>152,153</sup> Banzhaf-Strathmann *et al.*<sup>144</sup> also described the overexpression of DNMT3a in FTD brain samples, which might explain the hypermethylation state of both *GRN* and *C9orf72* genes. Other

**Table 1.** DNA (hydroxy)methylation alterations in dementia (human brain studies only)

Gene	Regulation	Disorder	Brain region	Methods	References
<i>ABCA7</i>	Increased 5mC	AD	Prefrontal cortex	BS array	57
<i>ANK1</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	WGBS, BS array, pyrosequencing	41,49,65
	Reduced 5mC	DLB	Prefrontal cortex	WGBS, BS array, pyrosequencing	41
<i>APP</i>	Increased 5hmC	AD	Prefrontal cortex	WG 5 hmC-enriched seq	89
	Reduced 5mC	AD	Parietal cortex, temporal lobe and cerebellum	MSRE-SB, pyrosequencing	51-53
<i>BDNF</i>	Increased 5mC	AD	Prefrontal cortex	MSRE-PCR	66
<i>BIN1</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	BS array	49,57
	Increased 5hmC	AD	Prefrontal cortex	WG 5 hmC-enriched seq	89
<i>C9orf72</i>	Increased 5mC	FTD	Frontal cortex, temporal cortex, motor cortex, cerebellum	MSRE-PCR, BS-seq, MSRE-SB	149,150,152
<i>COX2</i>	Reduced 5mC	AD	Prefrontal cortex	MSRE-PCR	49,66
<i>CREB</i>	Increased 5mC	AD	Prefrontal cortex	MSRE-PCR	66
<i>DIP2A</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	BS array	49
	Reduced 5hmC	AD	Prefrontal cortex	WG 5 hmC-enriched seq	89
<i>DNMT1</i>	Increased 5mC	AD	Prefrontal cortex	BS-MALDI	56
<i>DUSP22</i>	Increased 5mC	AD	Hippocampus	BS array, pyrosequencing	59
	Reduced 5hmC	AD	Prefrontal cortex	WG 5 hmC-enriched seq	89
<i>DUSP6</i>	Increased 5mC	AD	Superior temporal gyrus	BS array	60
<i>GPNMB</i>	Reduced 5mC	PD	Fronta cortex, cerebellum	BS array	115
<i>GRN</i>	Increased 5mC	FTD	Frontal cortex	BS-MALDI	144
<i>HLA-DRB5</i>	Increased 5mC	AD	Prefrontal cortex	BS array	57
<i>IGFBP7</i>	Reduced 5mC	AD	Prefrontal cortex	MeDIP	79
<i>MT-ND1</i>	Reduced 5mC	AD	Entorhinal cortex and substantia nigra	MeDIP	94
<i>MTHFR</i>	Increased 5mC	AD	Prefrontal cortex	BS-MALDI	56
<i>NF-kB</i>	Reduced 5mC	AD	Entorhinal and prefrontal cortex	BS array, MSRE-PCR	49,66
<i>PARK16/lq32</i>	Reduced 5mC	PD	Fronta cortex, cerebellum	BS array	115
<i>PSEN1</i>	Reduced 5mC	AD	Prefrontal cortex	BS-MALDI	56
<i>RHBDF2</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	WGBS, BS array, pyrosequencing	41,49
<i>SEPW1</i>	Reduced 5mC	DLB	Frontal cortex	ELISA, MS-PCR	112
<i>SERPINF1</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	BS array	49
<i>SERPINF2</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	BS array	49
<i>SLC24A4</i>	Increased 5mC	AD	Prefrontal cortex	BS array	57
<i>SNCA</i>	Reduced 5mC	PD	Substantia nigra and cortex	BS-seq, ELISA, MS-PCR	110-112
	Reduced 5mC	DLB	Frontal cortex	ELISA, MS-PCR	112
<i>SORBS3</i>	Increased 5mC	AD	Entorhinal and prefrontal cortex	BS array, pyrosequencing, MS-PCR	62,64
	Increased 5mC	AD	Prefrontal cortex	BS array	57
<i>SORT1</i>	Increased 5mC	AD	Frontal cortex	BS array, pyrosequencing	62
<i>STX1B</i>	Increased 5mC	PD	Fronta cortex, cerebellum	BS array	115
<i>TBXA2R</i>	Increased 5mC	AD	Frontal cortex	BS array, pyrosequencing, MS-PCR	62,64
<i>TMEM59</i>	Reduced 5mC	AD	Frontal cortex	BS array	58
<i>TREM2</i>	Increased 5mC	AD	Hippocampus	BS-seq	71
	Increased 5hmC	AD	Hippocampus	5hMeDIP	71

Abbreviations: AD, Alzheimer's disease; BS array, bisulfite-modified DNA based arrays; BS-MALDI, bisulfite matrix-assisted laser desorption/ionization; BS-seq, bisulfite sequencing; DLB, dementia with Lewy bodies; ELISA, enzyme-linked immunosorbent assay; FTD, frontotemporal dementia; 5mC, 5-methylcytosine; 5hmC 5-hydroxymethylcytosine; 5hMeDIP, 5-hydroxymethylated DNA immunoprecipitation; MeDIP, methylated DNA immunoprecipitation; MS-PCR, methylation specific PCR; MSRE-PCR, methylation-sensitive restriction enzyme PCR; MSRE-SB, methylation-sensitive restriction enzyme southern blot; PD, Parkinson's disease; WG 5 hmC-enriched seq, whole-genome sequencing analysis of 5-hydroxymethylcytosine-enriched sequences; WGBS, whole-genome bisulfite sequencing.

**Table 2.** GO analysis using the topGo tool for the genes with described epigenetic alterations in dementia

Ontology	GO terms	P-value	Genes
Biological process	Regulation of cellular component organization	4.4e-09	<i>ABCA7, ANK1, BIN1, DNMT1, GRN, IGFBP7, MTHFR, PSEN1, SERPINF1, SERPINF2, SNCA, SORBS3, SORL1, SPTBN4, STX1B, TMEM59, TREM2</i>
	Vesicle-mediated transport	9.1e-08	<i>ABCA7, ANK1, APP, BIN1, C9orf72, GRN, PSEN1, SERPINF2, SNCA, SORL1, SPTBN4, STX1B, TREM2</i>
	Regulation of protein modification process	2.4e-07	<i>ABCA7, APP, DNMT1, DUSP22, DUSP6, MTHFR, PSEN1, SERPINF2, SNCA, SORBS3, SORL1, SPTBN4, TMEM59, TREM2</i>
	β-Amyloid formation	7.0e-07	<i>ABCA7, PSEN1, SORL1</i>
	Negative regulation of protein metabolic process	8.2e-07	<i>ABCA7, APP, DNMT1, DUSP22, DUSP6, PSEN1, SERPINF1, SERPINF2, SNCA, SORL1, TMEM59</i>
	Negative regulation of proteolysis	1.6e-06	<i>APP, PSEN1, SERPINF1, SERPINF2, SNCA, SORL1, TMEM59</i>
	Regulation of cellular protein metabolic process	1.7e-06	<i>ABCA7, APP, DNMT1, DUSP22, DUSP6, MTHFR, PSEN1, SERPINF1, SERPINF2, SNCA, SORBS3, SORL1, SPTBN4, TMEM59, TREM2</i>
	Regulation of MAPK cascade	3.5e-06	<i>ABCA7, DUSP22, DUSP6, PSEN1, SERPINF2, SORBS3, SORL1, SPTBN4, TREM2</i>
	Positive regulation of protein metabolic process	3.6e-06	<i>ABCA7, APP, DNMT1, DUSP22, DUSP6, PSEN1, SERPINF2, SNCA, SORBS3, SORL1, SPTBN4, TREM2</i>
	Regulation of protein metabolic process	4.1e-06	<i>ABCA7, APP, DNMT1, DUSP22, DUSP6, MTHFR, PSEN1, SERPINF1, SERPINF2, SNCA, SORBS3, SORL1, SPTBN4, TMEM59, TREM2</i>
	Molecular function	Endopeptidase inhibitor activity	1.7e-04
Tau protein binding		1.8e-04	<i>BIN1, SNCA</i>
Endopeptidase regulator activity		1.9e-04	<i>APP, SERPINF1, SERPINF2, SNCA</i>
Peptidase inhibitor activity		2.0e-04	<i>APP, SERPINF1, SERPINF2, SNCA</i>
Enzyme binding		2.7e-04	<i>ANK1, APP, BIN1, C9orf72, DNMT1, SERPINF2, SNCA, SORL1, SPTBN4, STX1B</i>
Peptidase regulator activity		4.1e-04	<i>APP, SERPINF1, SERPINF2, SNCA</i>
Serine-type endopeptidase inhibitor activity		5.2e-04	<i>APP, SERPINF1, SERPINF2</i>
Structural constituent of cytoskeleton		6.3e-04	<i>ANK1, SORBS3, SPTBN4</i>
Spectrin binding		7.9e-04	<i>ANK1, SPTBN4</i>
Tyrosine/serine/threonine phosphatase activity		2.7e-03	<i>DUSP22, DUSP6</i>

Abbreviations: GO, gene ontology; MAPK, mitogen-activated protein kinase. The top 10 more significant terms at the biological process and molecular function characterization levels are shown. *P*-values show the statistical significance of the enrichment of gene ontology terms with analyzed genes.

genes associated with FTD, such as *APP*, *PSEN1* and *MAPT*, did not show any methylation differences in FTD brain samples compared with age-matched controls.<sup>54</sup>

The lack of any specific disease-modifying treatment for FTD makes it clear that more in-depth epigenetic analysis is necessary if we are to understand the role of DNA methylation as a potential driving event in this brain disorder. Genome-wide analysis of 5 mC and 5 hmC in different areas of post-mortem brain tissue will yield invaluable insights into potential new gene candidates that are epigenetically deregulated and simultaneously improve patient stratification, helping to differentiate FTD more accurately from other dementias.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Dementia covers a wide spectrum of cognitive abnormalities that arise as a clinical consequence of disorders such as AD, PD, DLB and FTLD. However, our knowledge of its etiology is rather limited and no effective treatments to prevent or revert this condition have been described so far. Dementias are age-associated disorders that, due to the progressive aging of our societies, are likely to increase in incidence.<sup>3</sup> Disorders such as AD, PD, DLB and FTLD not only share dementia as one of their core clinical manifestations but also have some molecular alterations in common, implying the existence of similar driving events that may diverge later, during the progression of each disorder. For example Aβ accumulations are considered a hallmark of AD but are also present in DLB; Tau hyperphosphorylation is observed not only in AD but also in PD and DLB; and α-synuclein inclusions are found in PD, DLB and AD.<sup>154–158</sup>

The importance of epigenetic mechanisms in learning and memory processes<sup>9,128,159</sup> has prompted the scientific community to analyze the role of the epigenome in neurological diseases.<sup>15</sup> Accordingly, it has been proposed (the LEARN hypothesis) that epigenetic alterations during the lifespan would disturb gene expression over the long term, in an accumulative way, acting as a 'seed' for the development of disorders such as dementia and neurodegeneration.<sup>7</sup> Thus, information about the epigenetic dysregulations of brain function would provide invaluable knowledge about the origin and progression of brain disorders, contributing to the development of effective therapeutic interventions. In this context, our laboratory has recently identified a network of genes undergoing an aberrant methylomic state of their promoters that is common to AD, PD and DLB.<sup>41</sup> While this is the first genome-wide demonstration of epigenetic alterations being shared by these disorders, several other alterations common to some of them have been described before (Tables 1 and 2). For example, *ANK1*, a gene associated with microglia activation, is epigenetically altered in AD<sup>41,49,65</sup> and DLB.<sup>41</sup> Other examples include the gene *SNCA*, which is aberrantly methylated in PD and DLB,<sup>112</sup> and *TREM2*, a gene mutated in FTD, which is epigenetically altered in AD.<sup>71</sup> In addition, α-synuclein can associate with DNMT1, sequestering it in the cytoplasm, resulting in global DNA hypomethylation. This effect of α-synuclein was found not only in PD cases but also in DLB.<sup>112</sup> As α-synuclein can also accumulate in AD,<sup>154</sup> this mechanism might also contribute to the global DNA hypomethylation seen in the latter. However, more in-depth analysis and genome-wide approaches are essential for understanding the role of aberrant epigenomes in dementia, especially in little understood disorders such as FTD and DLB. This is critical if we are to identify a causative role for DNA

methylation in the appearance of neurodegenerative diseases by proving that environmental factors (life experiences, toxins, other disorders) may alter our epigenome in an accumulative way that may eventually end by developing dementia (the LEARN hypothesis<sup>7</sup>).

Epigenetic brain changes are strongly correlated with normal aging,<sup>11,160,161</sup> suggesting that DNA methylation patterns are modified to start senescence programs.<sup>162</sup> Interestingly, accelerated epigenetic aging has been described in dementia-related disorders such as AD<sup>163</sup> and PD.<sup>164</sup> Together with the fact that age is the most important risk factor for many dementia-related disorders, these data suggest a strong association between dementia and an accelerated epigenetic aging.

The young field of neuroepigenomics<sup>13</sup> is increasingly identifying new druggable pathways and targets in dementia, demonstrating the importance of the epigenetic component in the healthy brain and in brain disorders.<sup>165</sup> The epigenetic discoveries made in the field of dementia in recent years suggest that neuroepigenomic alterations are fundamental in dementia. Several disorders that feature dementia share common aberrant DNA methylation patterns,<sup>41</sup> suggesting that these genes could be responsible for the common clinical symptoms presented. However, several methodological and biological drawbacks need to be addressed to properly understand the alterations of the epigenome that occur in dementia. This, together with more in-depth proper cross-disorder studies, will eventually open up the potential for designing effective therapeutic interventions to prevent or revert such pathologies. Until now, all human neuroepigenetic studies have been carried out using post-mortem brain tissue samples. Customarily, grey matter (or often a mixture of white and grey matter) is used for DNA extraction and DNA methylation changes are subsequently analyzed using bisulfite conversion or other techniques.<sup>166</sup> Furthermore, the heterogeneity of brain cells<sup>167,168</sup> makes more specific types of preparation essential if we are to understand the specific aberrant methylomes associated with dementia in particular cell populations. These limitations are probably the base of current discrepancies found in the literature on the epigenomics of dementia (different brain sections, even from the same brain area, thus different cellular composition; different 'life stages' of the patients, etc). Other possible confounders, such as geographic distribution of patients,<sup>169</sup> sex<sup>170</sup> and so on, may be important to explain discrepancies. On the other hand, future studies need to address properly the correlation between neuropathological stage of the disease studied and the changes in (hydroxyl)methylation to properly understand the pathological evolution of the disorder under the epigenomic point of view. Other critical issue is that classically, DNA methylation patterns have been interrogated through sodium bisulfite-treated DNA. This chemical agent transforms 'unprotected' cytosines to uracils. However, both methylation and hydroxymethylation modifications protect such cytosines to be transformed. Thus, bisulfite-based analysis do not allow to differentiate between 5 mC and 5 hmC, although the data generated on 5 hmC indicates the importance of this modification by itself,<sup>161</sup> especially in the brain.<sup>171</sup> Recently, a commercially available kit (TrueMethyl Array Kit (Cambridge Epigenetix, Cambridge, UK) has been developed allowing for the differentiation between 5 mC and 5 hmC.<sup>172</sup> Together with the development of new technologies for interrogating the epigenome (i.e. nanopore sequencing<sup>173</sup>), this kit will eventually increase the current knowledge of the role of 5-hydroxymethylation on dementia, and will force to revise previous published data on DNA methylation on brain disorders, possibly helping to understand current discrepancies. The epigenomic analysis of brain seeks to establish strong associations between the (hydroxyl)methylome and the human disorder, but those findings need to be validated to confirm if a specific alteration has a causative role on the disease. Together with the lack of longitudinal studies in human

samples that would allow to establish the disorder-causality role of the epigenetic alterations observed, it is clear that the need of better 'humanized' dynamic models of the pathologies could improve our understanding of dementia. Research with induced pluripotent stem cells differentiated to specific neuronal populations<sup>174</sup> or single-cell epigenomics<sup>175</sup> will undoubtedly reveal a completely new level of complexity and a set of genes that are altered in dementia-associated pathologies by identifying causal associations between neuronal dysfunctions, gene expression and epigenetic (de)regulation. Recent advances in genetic and epigenetic editing through technologies such as CRISPR/Cas9<sup>176,177</sup> will undoubtedly provide a powerful experimental tool that will allow to validate epigenetically altered targets to improve the possible causality of DNA methylome alteration in dementia.

The brain is an extraordinary organ showing high levels of epigenetic features, such as non-CpG methylation.<sup>21,178</sup> The understanding of the role of these non-CpG changes along with the more detailed dissection of the specific role of 5-methylcytosine, 5-hydroxymethylcytosine and newly discovered marks, such as the N6-methyladenine,<sup>95,179</sup> will help the scientific community understand the epigenetic mechanisms underpinning dementia. The recent analysis of human reference epigenomes<sup>180</sup> will enable future neuroepigenomics research to exploit the full potential of alterations in DNA methylation patterns in specific genes and cell populations to enable better patient diagnosis, and the stratification and treatment of dementia and other brain disorders.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### ACKNOWLEDGMENTS

We thank all the members of ME's laboratory and our collaborators for help and stimulating discussions. In particular, we would like to thank Dr Jose V Sanchez-Mut for his work in the laboratory during his PhD, Sebastián Morán and Manuel Castro for their help with the gene network analysis and Dr Joan Carles Balasch for his work on Figures 1 and 3. The work of ME's laboratory is supported by, among other institutions, the E-Rare (ERA-Net for research programs on rare diseases) and EuroRETT (a European network on Rett syndrome, funded by the European Commission under its 6th Framework Program since 2006); the EU Joint Programme—Neurodegenerative Disease Research (JPND; EPI-AD Consortium); Cellex Foundation; and the Health and Science Departments of the Catalan Government (Generalitat de Catalunya). ME is an ICREA Research Professor.

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