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Conserved and divergent features of DNA methylation in embryonic stem cellderived neurons — Source link \square

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1 Conserved and divergent features of DNA methylation in embryonic stem cell-derived

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26

27 Abstract

28 DNA methylation functions in genome regulation and is implicated in neuronal maturation. 29 Early post-natal accumulation of atypical non-CG methylation (mCH) occurs in neurons of 30 mice and humans, but its precise function remains unknown. Here we investigate mCH 31 deposition in neurons derived from mouse ES-cells in vitro and in cultured primary mouse 32 neurons. We find that both acquire comparable levels of mCH over a similar period as in vivo. 33 In vitro mCH deposition occurs concurrently with a transient increase in Dnmt3a expression, 34 is preceded by expression of the post-mitotic neuronal marker *Rbfox3* (NeuN) and is enriched 35 at the nuclear lamina. Despite these similarities, whole genome bisulfite sequencing reveals 36 that mCH patterning in mESC-derived neurons partially differs from in vivo. mESC-derived 37 neurons therefore represent a valuable model system for analyzing the mechanisms and 38 functional consequences of correct and aberrantly deposited CG and non-CG methylation in neuronal maturation. 39

40 Introduction

The unique epigenomic landscape of neurons is hypothesized to allow these postmitotic cells to respond to diverse environmental stimuli during development and to modify gene transcription in response to activity, while retaining their cellular identity (Cortes-Mendoza et al., 2013, Day et al., 2013, Feng et al., 2010, Graff et al., 2012, Miller and Sweatt,

45 2007, Stroud et al., 2017). DNA methylation is thought to play an important role in imparting this simultaneous robustness and adaptability to neurons (Bayraktar and Kreutz, 2018b, 46 47 Bayraktar and Kreutz, 2018a, Fasolino and Zhou, 2017). In most somatic cells, DNA 48 methylation is largely restricted to cytosines in the context of CG dinucleotides (mCG). The 49 methylation of CG sites is considered a relatively stable modification with a well-described 50 function in gene silencing and imprinting (Bird, 2002). In contrast, in adult mammalian brains, 51 other types of DNA methylation are found at high levels, including non-CG methylation (mCH, 52 where H = A, T, or C (Guo et al., 2014, He and Ecker, 2015, Lister et al., 2013, Xie et al., 53 2012) and intermediates in the DNA demethylation pathway. particularly 5-54 hydroxymethylcytosine (5hmC) (Kriaucionis and Heintz, 2009, Lister et al., 2013, Mellen et al., 55 2012, Szulwach et al., 2011). In adult human and mouse neurons up to ~50% of methylated 56 cytosines in the genome occur in the mCH context, a level similar to mCG (Guo et al., 2014, 57 Lister et al., 2013), and the majority of this exists in the mCA sequence context. While the 58 precise roles of these modifications are not fully understood, the complex and diverse 59 methylation profiles of adult neurons (Luo et al., 2017) suggest that DNA methylation plays an 60 important role in the dynamic and adaptable regulation of gene expression in these cells. 61 Studies in both mice and humans have shown that mCA is first observed in the brain shortly 62 after birth and continues to accumulate during development to adulthood, after which the 63 levels remain stable (Lister et al., 2013). This observation raises the exciting possibility that 64 the generation of mCA may link early life experiences with neuron function later in life. The level of intragenic mCA in neurons inversely correlates with transcript abundance (Mo et al., 65 66 2015, Stroud et al., 2017, Xie et al., 2012), and in mice mCA deposition is negatively regulated 67 by gene transcription (Stroud et al., 2017), suggesting that mCA functions as a part of a 68 molecular system to modulate gene expression in response to synaptic activity, and to 69 consolidate specificity of neuron subtypes.

DNA methylation is established and maintained by a family of conserved DNA
 (cytosine-5)-methyltransferases (DNMTs). Dnmt1 propagates existing methylation patterns at

72 symmetrically opposed CG sites during cell division and is essential for the maintenance of 73 methylation and chromosomal stability (Bayraktar and Kreutz, 2018a, Feng and Fan, 2009). 74 Dnmt3a and Dnmt3b, on the other hand, catalyse the *de novo* methylation of cytosine, and 75 the levels of Dnmt3a can be dynamically regulated to increase DNA methylation in the brain 76 (Feng et al., 2005). The post-natal deposition of mCH is driven by a transient increase in the 77 expression of Dnmt3a (Gabel et al., 2015, Guo et al., 2014, Lister et al., 2013, Luo et al., 2019, 78 Stroud et al., 2017), and conditional deletion of Dnmt3a in Nestin-positive neuronal precursors 79 during late gestation results in impaired motor activity (Nguyen et al., 2007). In contrast, 80 deletion of Dnmt3a in excitatory neurons at early postnatal stages was reported to have no 81 apparent major effect on brain development or function (Feng et al., 2010), suggesting that 82 the developmental window during which mCH is deposited is critical (Lister et al., 2013). The 83 importance of DNA methylation in governing correct neuronal function is exemplified by a 84 range of developmental neurological disorders that result from mutations in proteins 85 associated with DNA methylation in both the CG and CH context (Hamidi et al., 2015, lp et al., 86 2018).

87 Defining the roles of mCG and mCH in neuron maturation and synaptic plasticity is of fundamental importance for understanding normal and abnormal brain development, thus a 88 89 tractable and representative in vitro model system to further explore this process is highly 90 desirable. We therefore investigated the levels, distribution and temporal dynamics of mCH 91 during *in vitro* neuronal differentiation of human and mouse pluripotent stem cells. Deploying 92 a range of cellular and genomic assays, we reveal similar sub-nuclear patterning, levels, and 93 spatiotemporal dynamics of DNA methylome reconfiguration during the differentiation and 94 maturation of mouse neurons in vitro and in vivo, but also differences that likely arise from the complex influence of the *in vivo* cellular environment on neuron differentiation and maturity. 95

96

97 Results

98 Immunocytochemical labelling for mCA accumulates in post-mitotic neurons and 99 temporally correlates with DNMT3a expression.

100 In vivo, mouse cortical neurons begin to acquire readily detectable levels of mCH around 2 101 weeks after birth, which continues to increase up to 6 weeks of age and remains high 102 throughout adulthood (Lister et al., 2013). To assess whether this can be recapitulated in vitro. 103 we used two independent approaches. First, we isolated primary cortical and hippocampal 104 neurons from day 18 embryonic C57BL/6 mice (E18, average gestation 18.5 days) and 105 cultured these for up to 14 days in vitro (DIV). We hypothesized that if mCH accumulation was 106 due to cell intrinsic developmentally hardwired processes, 14DIV should correlate with the 107 temporal acquisition of mCH in vivo (Figure 1A). In the second approach, we adapted an 108 established differentiation protocol to generate mouse cortical neurons from embryonic stem 109 cells (mESCs) (Bibel et al., 2007). We hypothesized that if this developmental model 110 recapitulated neural development and neuronal maturation in vivo, mCH would occur within 111 several weeks (Figure 1B). Two different mouse ESC lines, R1 (Nagy et al., 1993) and G4 112 (George et al., 2007), were differentiated as cell aggregates for 8 days in suspension, followed 113 by dissociation and continued differentiation in adherent culture for up to 30 additional days to 114 yield mixed cultures enriched in post-mitotic neurons (Supplementary Figure 1A). Both cell 115 lines developed mature neurons within an equivalent time course and to a similar extent, as 116 assessed by morphology and immunohistochemistry (R1 and G4), TEM analysis of synaptic 117 depolarization (R1), and c-Fos mRNA and ICC analysis following depolarization (G4) 118 (Supplementary Figure 1). To investigate the temporal acquisition, sub-nuclear localisation, 119 and cell-type specificity of mCH in cultured neurons, we used an antibody raised against the 120 mCA dinucleotide (anti-mCA) to analyse the primary- and mESC-derived neuronal cultures by 121 immunocytochemistry. To identify neurons, cultures were co-labelled for NeuN/Rbfox3, a well-122 established marker of most postmitotic neuron subtypes, and beta3-tubulin (TUBB3), a pan-123 neuronal marker. Specificity of the mCA antibody was confirmed using a panel of competitive 124 methylated oligonucleotides (Figure 1C – 1F, Supplementary Figure 2).

Martin et al., Figure 1



Figure 1 In vitro acquisition of DNA methylation in primary and ESC-derived mouse neurons

128 (A, B) Timeline schematic of neuron development in mouse cortex (A) and from mESCs in 129 vitro (B). (C-F) 14DIV cortical (C, D) or hippocampal (E, F) neurons immunolabelled for NeuN, 130 TUBB3 and mCA \pm 2.5 μ M [5mC]A. Scale bar = 50 μ m. Analysis of the relative intensity of 131 NeuN and mCA fluorescence in DAPI-masked nuclei from 14DIV cortical (D) or hippocampal 132 (F) neurons. (G, H) mESC-derived neurons were fixed at different times during maturation and 133 labelled for NeuN, mCA and DAPI. Scale bar = 20 µm. The nuclear fluorescence intensity (AU) 134 of NeuN and mCA was determined. Results = mean \pm SEM, for one representative 135 differentiation. Similar labelling profiles were observed in 3 separate differentiations. (I, J) 136 Neural progenitors (Day 9-10, Pax6+ve) and neurons (Day 20-40 NeuN+ve) were 137 immunolabeled for mCA, and level of mCA nuclear fluorescence intensity quantified. Results 138 = mean \pm SD, n=3 separate differentiations for Pax6 and 2 differentiations for NeuN. Scale 139 bar = 10 μ m. (K, L) Later stage neural differentiations (Day 30-38) were immunolabeled for 140 astrocytes (GFAP), neurons (TUBB3) and mCA. Nuclei were manually masked and the level 141 of mCA measured in TUBB3+ve neuronal cells, GFAP+ve astrocytes, and GFAP-ve/TUBB3-142 ve cells of unknown identity. Quantitation shown was acquired from a single late-stage 143 differentiation and labelling averaged across 5 random fields of view. Results are the mean ± 144 SD. Arrows mark nuclei of astrocytes (red), neurons (white), and other cell types (yellow). Scale bar = 50 µm. (M) Gene expression of Dnmt1, Dnmt3a and Rbfox3 (NeuN) were 145 146 determined by RT-gPCR relative to beta-actin. Results = mean ± SEM, n=3-4. For all 147 experiments, statistical analysis was performed using a Student's t-test. * p <0.01, ** p<0.05, *** p <0.001, **** p <0.0001. 148

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Martin et al. Supplementary Figure 1



152 Supplementary Figure 1 Characterisation of mESC-derived neurons

(A) Phase contrast images of mESCs growing on a layer of feeder MEFs, cell aggregates (day
6), neural progenitors (day 9), and neurons (day 18-38). Scale bar =100 μm, except for cell

155 aggregates = 200 µm. (B) Neurons derived from either G4 mESCs or R1 mESCs were fixed 156 and labelled for the pan-neuronal marker beta3-tubulin (TUBB3). Both cell lines generated 157 complex neurite networks within 38 days. Scale bar = 100 μ m. (C) Neurons derived from G4 158 mESCs were fixed and labelled for TUBB3 and the pre-synaptic protein synapsin 1. Punctate 159 labelling for Syn1 along neurites demonstrates the presence of nascent synapses. Scale bar 160 = 50 μ m. (D) The response of synapses to depolarisation was shown by transmission electron 161 microscopy. R1 mESC-derived neurons were incubated for 5 min in the presence of CTB-HRP 162 in either low K^+ or high K^+ buffer. Low levels of tracer endocytosis into synaptic vesicles in low 163 K^+ was superseded by high levels of bulk endocytosis in depolarised cells, suggesting a strong 164 rapid burst of neuroexocytosis and compensatory endocytosis (Cousin, 2009). (E) G4 mESC-165 derived neurons were fixed and labelled for TUBB3 at different time points during 166 differentiation. Scale bar = $20\mu m$. (F) G4 mESC-derived neurons were depolarised for 5 min 167 with high K^+ , then chased in growth medium for between 1h and 6 h. Cells were fixed and 168 labelled for c-Fos and TUBB3. The intranuclear intensity (IntDen) of c-Fos was determined 169 during the chase. Results = mean \pm SEM, for one representative differentiation. Similar 170 labelling profiles were observed in two separate differentiations. Depolarisation resulted in a 171 transient increase in intranuclear c-Fos labelling in the post-depolarisation period. **** p 172 <0.0001, Student's t-test. Scale bar = 50 µm. (G) c-Fos mRNA abundance was determined by 173 RT-qPCR in control G4 mESC-derived neurons and during a 24 h chase following a 5 min 174 transient depolarisation by high K^+ . Results shown are mean \pm SEM relative to beta-actin 175 levels. N=3 independent experiments. * p <0.05, ** p <0.01, Student's t-test.

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Martin et al. Supplementary Figure 2

179 Supplementary Figure 2 Specificity of the anti-mCA antibody determined by ICC

180 **(A)** mESC-derived neurons were fixed and immunolabelled for NeuN and mCA \pm 2.5 μ M 181 competitive methylated oligonucleotides: [5mC]A, [5mC]G, [5mC]T and [5mC]C, or the non-182 methylated CA oligonucleotide. Scale bar = 10 μ m. **(B)** The Integrated Density (IntDen) of the 183 nuclear mCA labelling in NeuN-masked nuclei was determined. **(C)** Sequence of the 184 methylated oligonucleotides.

185

To first determine whether *in vitro* cultured neurons could acquire non-CG methylation, 14DIV cortical and hippocampal primary neuronal cultures were immunolabelled for mCA, NeuN, and TUBB3 (Figure 1C, E). At this DIV, the majority of cells displayed a strong intranuclear labelling for mCA, in addition to labelling for both neuronal markers. Nuclear labeling for mCA was completely abrogated by the competitive [5mC]A oligonucleotide (Figure 1C-F, Supplementary Figure 2) confirming the specificity of the antibody. To quantify mCA labelling,

192 cell nuclei were masked using DAPI fluorescence and the level of mCA and NeuN 193 immunofluorescence in individual nuclei measured. The relative levels of mCA to NeuN were 194 then determined (Figure 1D, 1F). Consistent with the enrichment of mCA in neurons, we found 195 a strong positive correlation between the level of NeuN labelling and the level of mCA labelling 196 in both hippocampal (r=0.62) and cortical (r=0.83) cells.

197 We next used the anti-mCA antibody to analyse the accumulation of mCA at different 198 times up to 38 days during the differentiation and maturation of the mESC-derived neurons 199 (Figure 1G, H). Neuronal cells expressed TUBB3 one day after attachment (day 9, 200 Supplementary Figure 1E), and detectable NeuN labelling was observed within 3-6 days of 201 attachment (differentiation day 11-14, Figure 1G), suggesting the rapid development of a post-202 mitotic phenotype. Interestingly, this temporally correlates with the initial identification of NeuN 203 at E10.5 in the embryonic mouse brain (Mullen et al., 1992), suggesting that these 204 developmental milestones are temporarily hardwired and can be recapitulated in vitro. 205 Consistent with the early development of post-mitotic neurons, there was no further increase 206 in the number of NeuN-positive neurons over the 4 weeks in adherent culture, although there 207 was an obvious but variable increase in the number of non-neuronal cells within this time, 208 including glial cells (see Figure 1K), as described previously (Bibel et al., 2004). 209 Immunocytochemical (ICC)-based analysis of DNA methylation in NeuN-positive cells 210 revealed an increase in the level of nuclear mCA labeling between days 18 and 28, which 211 remained high to day 38 (Figure 1G, H). As the initial observation of mCA was significantly 212 later than the initial observation of NeuN, a post-mitotic phenotype is likely a prerequisite for 213 subsequent acquisition of mCA. Consistent with this, we found only minimal labelling for mCA 214 in Pax6-positive neural progenitors differentiated for 9-10 days relative to ~2-fold higher levels 215 in NeuN-positive neurons differentiated for 28-38 days (Figure 11, J). Similarly, we found 216 minimal labelling for mCA in GFAP-positive glial cells and in additional unidentified cell types 217 within the cultures that did not label for either neuronal or glial markers (Figure 1K, L).

218 Methylation of CH sites has been shown to be catalysed by Dnmt3a in vivo (Feng et 219 al., 2005, Stroud et al., 2017). We therefore analysed the transcript abundance of Rbfox3 220 (NeuN) and the DNA methyltransferases Dnmt3a and Dnmt1 during differentiation by RT-221 aPCR (Figure 1M). Consistent with the results of the ICC, Rbfox3 (NeuN) expression was found to significantly increase between days 9 and 18, reaching a plateau around day 28. In 222 223 contrast, and in agreement with previous data from both primary neurons and mouse brain 224 (Feng et al., 2005, Lister et al., 2013), Dnmt3a expression transiently increased between days 225 18 and 28 of differentiation and decreased again by 38 days. This transient increase in Dnmt3a 226 transcript level temporally correlates with the accumulation of mCA labelling observed by ICC 227 in the neurons (Figure 1G), strongly supporting the prediction that Dnmt3a catalyses CA 228 methylation in the mESC-derived neurons. Dnmt1 expression was initially high (Day 9) but 229 rapidly decreased after cell attachment, consistent with a role in the maintenance of DNA 230 methylation in the mitotic neural precursors. Collectively these data demonstrate that mCA 231 deposition occurs specifically in post-mitotic neurons but not in non-neuronal cell types and 232 correlates with DNMT3a expression.

233

234 mCA associates with the nuclear lamina in neurons

During our ICC analysis of mCA labelling, we observed that this DNA modification was enriched near the nuclear periphery in our *in vitro* neuronal cultures. To determine whether this localisation was unique to mCA, ESC-derived neurons were co-labelled for total 5methylcytosine (5mC) using an antibody predicted to label both mCG and mCH, and with the mCA-specific antibody, and the intranuclear distribution of the two compared (Figure 2A).



Martin et al., Figure 2

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243 **(A)** mESC-derived neurons were immunolabelled for total 5mC (mCG + mCH), mCA, and 244 NeuN, and nuclei identified using DAPI. Scale bar = 10μ m. **(B)** mESC-derived neurons were 245 immunolabelled for NeuN and either mCG, mCA, or 5hmC. Scale bar = 10μ m. **(C)** Primary

mouse hippocampal and cortical neurons (14DIV) were labelled for mCA and TUBB3. Scale bar = $20\mu m$. (**D**) Immunohistochemical analysis of DNA methylation in adult mouse hippocampus and cortex immunolabeled for either mCG or mCA, and NeuN, and nuclei identified using YOYO1. Scale bar = $20 \mu m$.

250 Consistent with a more restricted intranuclear distribution of mCA, total 5mC labelling was 251 observed to be more broadly distributed within the nucleus. While both marks showed a diffuse 252 labeling, mCA was highly enriched at the nuclear periphery, whereas 5mC additionally strongly 253 labelled intranuclear foci, which were devoid of mCA labelling. The intensely labelled 5mC foci 254 were found to also stain strongly with DAPI (Figure 2A) suggesting regions of tightly packed 255 heterochromatin that exclude mCA methylated DNA, in agreement with previous studies in 256 mouse brain (Lister et al., 2013, Stroud et al., 2017).

257 To directly examine the distribution of DNA methylated in specific sequence contexts. 258 neurons were labelled for either mCA or using an antibody specific for mCG (Figure 2B). In 259 addition, we examined localisation of 5-hydroxymethylcytosine (5hmC) (Figure 2B), which is 260 also highly enriched in neuronal genomic DNA (Kriaucionis and Heintz, 2009, Mellen et al., 2012, Szulwach et al., 2011). This labelling confirmed that the intense DAPI-stained foci 261 262 observed in Figure 2A were enriched in mCG labelling. Again, mCA was enriched at the 263 nuclear periphery, suggesting an association with the nuclear lamina. 5hmC was diffusely 264 distributed throughout the nucleus, consistent with an enrichment in euchromatin (Chen et al., 265 2014).

To confirm that the distribution of mCA observed *in vitro* faithfully represented the intranuclear localisation *in vivo*, we analysed the localisation of mCA in primary neurons cultured for 14DIV (Figure 2C), and of mCA and mCG in immunohistochemical sections of adult mouse brain (Figure 2D). Both primary neurons and cortical or hippocampal brain sections showed an identical enrichment of mCA labelling at the nuclear periphery.

271 Collectively these data show that DNA methylated in the CA context associates with the 272 nuclear lamina in neurons.

273

mESC-derived neuronal cultures acquire mCH to levels similar to those observed in vivo.

Having shown that both mESC-derived neurons and cultured primary neurons acquired mCA labeling using immunocytochemistry, we next determined global levels of DNA methylation (CG and CH) by whole-genome bisulfite sequencing (WGBS).

279 In primary neurons, we found very low levels of mCH (<0.1%) at E18 in cells isolated 280 from either the hippocampus or the cortex, consistent with previous in vivo data (Lister et al., 281 2013) and E12.5 mouse NPCs cultured in vitro (Luo et al., 2019). Following 14DIV, this level 282 increased to 0.70% and 0.79% in primary cortical and hippocampal neurons, respectively 283 (Figure 3D), confirming that the mechanisms underpinning accumulation of mCH are 284 conserved in *in vitro* cultures. This is in agreement with the *in vitro* differentiation of E12.5-285 derived mouse NPCs, which also show an increase in mCH levels over several weeks of 286 culture, reaching a maximum of 0.35% mCH/CH after 21 days (Luo et al., 2019). Analysis of 287 mCH context confirmed that methylation at CA sites was the most abundant modification, 288 although smaller increases in methylation in the CT and CC contexts were also observed 289 (Figure 3E-G). Comparison of the methylation levels at 14DIV to those of 2 week old mouse 290 prefrontal cortex (PFC) (Lister et al., 2013) showed that these were similar for all three mCH 291 subtypes. In contrast, the levels of mCG were slightly higher in vitro than in vivo (Figure 3C).



Martin et al., Figure 3

292

293

Figure 3 WGBS analysis of DNA methylation in primary mouse neurons and mESCderived neurons

(A, B) Timeline schematic of neuron development in mouse cortex (A) and from mESCs in
vitro (B). Time points analysed by WGBS are highlighted red. (C-G) Global levels of DNA
methylation in mouse brain ((Lister et al., 2013), dark blue bars), primary mouse cortical or
hippocampal neurons (E18 and 14DIV, purple bars) and mESC-derived neuronal cultures (R1

and G4 cell lines, light blue bars). Values represent the weighted methylation levels: the fraction of all WGBS base calls that were C at cytosine positions in the genome (for each context separately). Results = single samples, except 14DIV where n=2, mean \pm SD.

303

304 Analysis of global DNA methylation levels by WGBS was then undertaken on the 305 mESC-derived neuronal cultures between days 27 and 38 post-differentiation, which are 306 temporally comparable to approximately 1 week and 3 weeks post-natal in vivo development, 307 respectively (Figure 3A, B). Consistent with the primary neuron analysis, both the G4- and R1-308 derived neuronal cultures acquired high levels of mCH within 27 days, consisting 309 predominantly of mCA. The global level of mCH and mCA was very similar between the two 310 cell lines and showed a time-dependent increase up to 38 days. Global levels of mCA and 311 mCH at 38 days (equivalent to ~3 weeks post-natal in vivo) closely mirrored those of the 312 prefrontal cortex of 2 week old mice (Figure 3D, E), although the levels at 27 days (~1 week 313 post-natal in vivo) were ~5-fold higher than in 1 week old mouse PFC. Whether this reflects 314 an earlier deposition of mCH in vitro or is a result of differing proportions of neuronal and non-315 neuronal cells in the two sample types is not known. Smaller increases in the level of mCT 316 and mCC were also observed in vitro, as well as an increase in the level of mCG. Our data 317 indicate that in vitro neuronal differentiation of mESCs recapitulates overall in vivo levels of 318 mCH and mCA, which is distinct from prior report of low levels of mCH and mCA in iN cells 319 (Luo et al., 2019). Thus, we conclude that acquisition of mCH and mCA is largely a neuro-320 developmentally hardwired process.

321

322 Global mCH and mCG levels in mESC-derived neurons reveals hypermethylation 323 relative to *in vivo* adult neurons.

324 As mESC-derived neuronal cultures contain multiple cell types (Figure 1) fluorescence-325 activated nuclear sorting (FANS) was used to analyse mCG and mCH levels in specific 326 populations of cells (Figure 4A, B). To analyse the developmental timeline of mESC-derived 327 neurons we sorted Nanog-positive nuclei from mESCs, Pax6-positive nuclei from mESC-328 derived neuronal progenitor cells, NeuN-positive nuclei from cells cultured for 30 days, and 329 NeuN-positive and NeuN-negative nuclei from cells cultured for 38 days (Figure 4A, see also 330 Figure 1A, B). To investigate a possible temporal regulation of methylation patterns we also 331 isolated NeuN-positive cells from various human ESC-derived neuronal cultures, including 332 both 2-D cortical differentiation (Reinhardt et al., 2013) and 3-D cerebral organoids (Lancaster 333 and Knoblich, 2014) (Figure 4B). We then analysed by WGBS the levels of mCG and mCH in 334 these different nuclear populations, representing different cell types within the various 335 neuronal differentiation timelines (Figure 4A and 4B).

336 During the mouse ESC differentiation process, global CG methylation levels were 337 observed to progressively increase, with the highest increment observed during the maturation 338 of Pax6-positive neural progenitors to 30-day old neuronal nuclei, corresponding to the period 339 during which maximal Dnmt3a transcript abundance was observed in bulk cultures (Figure 340 1M). The observed levels of mCG in NeuN-positive nuclei, at both 30 days and 38 days, was 341 substantially higher than that reported in NeuN-positive nuclei isolated from mouse PFC, and 342 was higher than the equivalent NeuN-negative cells in the cultures, suggesting specific 343 hypermethylation of neuronal CG occurs *in vitro*. Interestingly, hypermethylation of CG was 344 not observed in the human cultures, where we observed generally lower global levels of mCG 345 than in vivo (for four out of five cultures).

346

Martin et al., Figure 4



347

348 Figure 4 DNA methylation levels in fluorescence-activated sorted nuclei

349 (A, B) Schematics showing time points during the mESC and hESC differentiation to neurons 350 at which nuclei were isolated. (A) Samples from the mESC differentiation included Nanog +ve 351 mESCs, Pax6 +ve neural progenitors, day 30 and 38 NeuN +ve mouse neurons and day 38 NeuN -ve cells. (B) In the human ESC differentiation, NeuN +ve nuclei were isolated following 352 353 12-16 weeks of 2-D culture, or 6-9months of 3-D culture. (C-G) Level of DNA methylation in 354 isolated nuclear populations. Light blue (mouse), red (human 2-D) and pink (human 3-D) bars 355 show samples generated in this study. Dark blue (mouse) and yellow (human) bars show 356 previously published levels of DNA methylation (Lister et al., 2013).

357

358 We next analysed the levels of mCH in the various nuclei populations. In the mouse samples. 359 the level of mCH was found to increase substantially during the transition from Pax6-positive 360 NPCs to 30 day old NeuN-positive neurons, and again between day 30 and day 38 of neuron 361 maturation (Figure 4D). The levels attained by day 38 of culture exceeded that of 7 week old 362 mouse prefrontal cortex (approximately 65 days total development from the blastocyst stage), 363 suggesting that for mCH, as for mCG, hypermethylation of the *in vitro*-derived neurons was 364 occurring. This pattern of methylation was recapitulated for the individual analyses of mCA, 365 mCC and mCT (Figure 4E-F). For mCA, we found a level of 1.25% (mCA/CA) in Nanog-366 positive mESC-derived nuclei, consistent with published studies in mice (Arand et al., 2012, 367 Ramsahoye et al., 2000) and humans (Liao et al., 2015, Ziller et al., 2011). This level was 368 found to decrease in Pax6-positive neural progenitors, and subsequently increased to 2.9% 369 (day 30) and 5.3% (day 38) in NeuN-positive nuclei. As with mCG and mCH, at day 38 this 370 level was higher than that reported in NeuN-positive nuclei isolated from mouse brain, again 371 suggesting hypermethylation of CA. Consistent with the ICC results and published in vivo data 372 (Lister et al., 2013), NeuN-negative cells within the 38-day old neuronal cultures contained low 373 levels of mCA. The levels of mCT in the day 38 ESC-derived neurons reached levels similar 374 to those in the adult mouse brain, while only negligible levels of mCC were detected in any 375 cell type. Together these data demonstrate that mESC-derived neurons acquire non-CG 376 methylation levels similar to in vivo levels.

In contrast to mouse ESC-derived neurons, only negligible levels of non-CG methylation were observed in any of the human ESC-derived neuronal populations, suggesting that even temporally extended human neuronal cultures are unable to mature sufficiently to acquire mCH. No difference in mCH was detected between shorter, 2-D neuronal cultures, and aged cerebral organoids, suggesting that the culture conditions alone do not promote the acquisition of mCH.

383

384 Genome wide distribution of mCH and mCG DNA methylation between *in vivo* and 385 mESC-derived neurons indicates regional hypermethylation

To establish the degree to which DNA methylation patterns in the ESC-derived neurons recapitulated those of *in vivo* neurons, we generated base resolution methylomes by WGBS and assessed the regional distribution of mCG (Figure 5) and mCH (Figure 6). We then compared this to previously published datasets from 7-week old mouse PFC glial cells (glia) or NeuN-positive neurons (*in vivo* neurons), and fetal mouse frontal cortex (fetal)(Lister et al., 2013), in order to identify potential differences between *in vivo* and *in vitro* neurons (Figures 5 and 6) and the similarities (Figure 7).

Martin et al., Figure 5



395 Figure 5 Global mCG properties of in vivo vs in vitro generated neurons

396 mCG characteristics for d38 mESC-derived neurons (in vitro neurons), 7-week old mouse 397 prefrontal cortex neurons (in vivo neurons), NeuN-negative cells from 7-week old mouse 398 prefrontal cortex (glia), and fetal mouse frontal cortex (fetal). (A) Weighted methylation levels 399 (mCG/CG) for all genes and 10 kb flanking regions shown at top. Heatmap shows mCG of 400 genes and flanking regions sorted by difference in gene body mCG/CG between mESC-401 derived neurons and in vivo adult mouse PFC neurons, as indicated on the right of the 402 heatmap. (B) Weighted CG methylation level throughout CpG islands (CGIs) for all CGIs and 403 10 kb flanking regions. (C) Weighted methylation levels (mCG/CG) for the whole genome, 404 intergenic regions, introns, exons, CGIs, and 500 bp flanking transcription start sites (TSS). 405 (D) Hierarchical clustering based on Spearman correlation of mCG levels in all 10 kb bins of

406 the genome. **(E)** Enriched pathways after pre-ranked gene set enrichment analysis based on 407 differences in gene body mCG/CG between in vivo and in vitro neurons. Shown are top 408 pathways based on enrichment scores (NES) for genes with higher mCG/CG in vitro (positive 409 NES score) and lower mCG/CG in vitro (negative NES score).

410

411 Analysis of average mCG levels across all gene bodies and associated 10 kb flanking 412 regions (Figure 5A) showed that these were generally similar between fetal, glial and in vivo 413 adult neurons, but exhibited a generalised increase in ESC-derived neurons (designated as 414 in vitro neurons) (Figures 5A, top plots), consistent with the observed global mCG levels 415 (Figure 4). Analysis of mCH levels (Figure 6A) showed that these were very similar between 416 in vivo and in vitro neurons, although there was a small localised increase around transcription 417 start sites *in vitro*, and they were much higher than either the glial or fetal samples (Figure 6A, 418 top plot). Genes were subsequently ordered by difference in mean gene body methylation 419 level relative to in vivo neurons (Figure 5A and 6A, lower heatmaps). In this analysis, we 420 observed that 38.7% of genes were mCG hypermethylated (gene body Δ mCG > 0.1) in *in vitro* 421 neurons compared to in vivo neurons, while only 0.6% of genes showed mCG 422 hypomethylation (Δ mCG > 0.1). In the CH context the ratio was more balanced, with 16.0% 423 of genes hypermethylated with Δ mCH > 0.01, and 12.2% of genes hypomethylated with Δ mCH 424 > 0.01. Thus, while global and average mCG and mCH gene methylation levels are similar 425 between *in vitro* and *in vivo* neurons, differences in the level of gene body DNA methylation 426 are evident. As a recent study had described DNA methylation patterns in directly 427 reprogrammed mouse neurons (iN cells) (Luo et al., 2019), we also compared our gene body 428 methylation data to the iN cell data (Supplementary Figures 3 - 4). This analysis showed that 429 the hypermethylation of mCG observed in mESC-derived neurons was unique to this 430 developmental model, as both the levels and the patterns observed in iN cells were very similar 431 to those of the *in vivo* neurons (Supplementary Figure 3). For mCH, the overall level of gene 432 body methylation in iN cells was very low (mCH/CH) and lacked the localised spike in

433 methylation observed at TESs in vivo and in ESC-derived neurons (Supplementary Figure 4). 434 To directly compare methylation patterns and compensate for the lower overall mCH levels, 435 gene body methylation levels were normalised to the average global methylation level within 436 individual neuron populations. For mCG, despite the slightly lower methylation levels in the iN 437 cells, when methylation patterns were normalised, a higher degree of similarity was observed 438 to the mESC-derived neurons, suggesting that neither *in vitro* neuron population attains genic 439 mCG patterning identical to in vivo neurons (Supplementary Figure 3). For mCH, more 440 complex differences were observed, with both iN cells and ESC-derived neurons showing 441 regional variation in the degree of similarity to *in vivo* neurons (Supplementary Figure 4).

Martin et al., Supplementary Figure 3



mCG over all gene bodies, genes sorted by difference in mCG between neurons

443

444 Supplementary Figure 3 DNA methylation in CG context in gene bodies in ESC-derived

445 and iN cells compared to in vivo neurons

Genes in the same order based on CG methylation difference as in Fig 5A but showing CG methylation for gene bodies and flanking 10 kb for 7-week adult mouse prefrontal cortex neurons (in vivo neurons), mESC-derived neurons (in vitro neurons) and trans-differentiated neurons (iN cells) as reported by (Luo et al., 2019). Left side = original data, right side = normalized to average global CG methylation levels.

Martin et al., Supplementary Figure 4



mCH over all gene bodies, genes sorted by difference in mCH between neurons

452

453 Supplementary Figure 4 DNA methylation in CH context in gene bodies in ESC-derived

454 and iN cells compared to in vivo neurons

Genes in the same order based on CH methylation difference as in Fig 6A but showing CH methylation for gene bodies and flanking 10 kb for 7-week adult mouse prefrontal cortex neurons (in vivo neurons), d38 mESC-derived neurons (in vitro neurons) and transdifferentiated neurons made from fibroblasts (iN cells) as reported by (Luo et al., 2019). Left side = original data, right side = normalized to average global CH methylation levels.

460



Martin et al., Supplementary Figure 5

463

464 Supplementary Figure 5 DNA methylation in CH context in gene bodies sorted for 465 differences in mCG between neuronal samples

Genes in the same order based on CG methylation difference as in Fig 5A but showing CH methylation for gene bodies and flanking 10 kb for fetal mouse frontal cortex (fetal), NeuNnegative cells from 7-week adult mouse prefrontal cortex (glia), 7-week adult mouse prefrontal cortex neurons (in vivo neurons), and d38 mESC-derived neurons (in vitro neurons). Difference in mCG between both neuronal samples used for gene order is shown on the right

coverage over all gene bodies, genes sorted by difference in mCG between neurons higher mCG in vitro fetal glia in vivo neurons in vitro neurons vitro Cof mCG in 10 average no. lower 10kb TSS TES +10kb -10kb TSS TES +10kb -10kb TSS TES +10kb 10kb TSS TES +10k ∆mCG 12 10 average no. of sequenced Cs 4

Martin et al., Supplementary Figure 6

-10kb TSS TES +10kb -10kb TSS TES +10kb -10kb TSS TES +10kb -10kb TSS TES +10kb

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474 Supplementary Figure 6 Cytosine coverage in gene bodies sorted for differences in

475 *mCG between neuronal samples*

476 Genes are in the same order based on CG methylation difference as in Fig 5A but showing

477 average number of covered cytosines per bin for gene bodies and flanking 10 kb for fetal.

478 mouse frontal cortex (fetal), NeuN-negative cells from 7-week adult mouse prefrontal cortex

(glia), 7-week adult mouse prefrontal cortex neurons (in vivo neurons), and d38 mESC-derived
neurons (in vitro neurons). Difference in mCG between both neuronal samples used for gene
order is shown on the right.

482

483 To investigate the relationship between mCG and mCH within gene bodies in the ESC-484 derived neurons, we assessed mCH patterns over all genes ordered by gene body mCG 485 difference (in vitro neuron mCG - in vivo neuron mCG; Supplementary Figure 5), and gene 486 body mCG patterns over all genes ordered by gene body mCH difference (in vitro neuron mCH 487 - in vivo neuron mCH; Supplementary Figure 7). These analyses revealed that the patterns of 488 DNA methylation levels observed were different for each context (Figure 5A and 6A). Indeed 489 a very low Pearson correlation of differences in methylation for both contexts between ESC-490 derived neurons and in vivo neurons (r = 0.0688) suggests that independent processes define 491 DNA methylation levels for each context, and that individual genes are methylated to a 492 different degree by mCG and mCH, compared to the global average levels. Plotting the total 493 number of calls at cytosine reference positions within gene bodies in the same order confirmed 494 that the observed differences are not a result of different sequencing coverage between 495 samples (Supplementary Figures 6 and 8).

496 In mESC-derived neurons, a marginal and consistent increase in mCG was observed 497 within and flanking CpG islands (CGIs), regions that are normally depleted of methylation 498 (Schubeler, 2015) (Figure 5B). Similarly, there was a slight increase in mCH methylation within 499 CGIs. However, there was a pronounced and localised increase in the level of mCH in the 500 regions immediately flanking CGIs (Figure 6B), suggesting that methylation of CG and CH 501 sites in these regions are regulated differently. In order to assess whether particular genomic 502 features exhibited different methylation levels in mESC-derived neurons, we measured the 503 weighted methylation levels (mCG/CG or mCH/CH) in intergenic regions, introns, exons, and 504 500 bp upstream and downstream of transcription start sites (Figures 5C and 6C). This

revealed genome-wide CG hypermethylation in mESC-derived neurons (~6-12% higher than in *in vivo* neurons, absolute methylation level difference), suggesting a generalised dysregulation of methylation level, while increased mCH was observed in all genomic features except exons. The reason for the specific exclusion of exons from hypermethylation in the mCH context is unknown but suggests that specialised mechanisms regulating exonal mCH levels are conserved *in vitro*.



Martin et al., Figure 6

512 Figure 6 Global mCH properties of in vivo vs in vitro generated neurons

511

513 mCH characteristics for d38 mESC-derived neurons (in vitro neurons), 7-week old mouse 514 prefrontal cortex neurons (in vivo neurons), NeuN-negative cells from 7-week old mouse 515 prefrontal cortex (glia), and fetal mouse frontal cortex (fetal). **(A)** Weighted methylation levels 516 (mCH/CH) for all genes and 10 kb flanking regions shown at top. Heatmap shows mCH of 517 genes and flanking regions sorted by difference in gene body mCH/CH between mESC-518 derived neurons and in vivo adult mouse PFC neurons, as indicated on the right of the 519 heatmap. (B) Weighted CH methylation level throughout CpG islands (CGIs) for all CGIs and 520 10 kb flanking regions. (C) Weighted methylation levels (mCH/CH) for the whole genome, 521 intergenic regions, introns, exons, CGIs, and 500 bp flanking TSSs. (D) Hierarchical clustering 522 based on Spearman correlation of mCH levels in all 10 kb bins of the genome. (E) Enriched 523 pathways after pre-ranked gene set enrichment analysis based on differences in gene body 524 mCH between in vivo and in vitro neurons. Shown are top pathways based on enrichment 525 scores (NES) for genes with higher mCH/CH in vitro (positive NES score) and lower mCH/CH 526 in vitro (negative NES score).

527

528 Next, we assessed regional correlation in mCG and mCH levels in 100 kb bins of the 529 whole genome (excluding chromosomes X and Y) between mESC-derived neurons, fetal 530 frontal cortex, and 7-week old mouse PFC neurons and glia (Figure 5D and 6D). For mCG, 531 fetal brain and adult neurons were the most similar, with adult glia joining at the next node. 532 while mESC-derived neurons formed their own branch. The low correlation between neuronal 533 samples is likely due to the overall higher methylation of CG in ESC-derived neurons. For 534 mCH, there was a high similarity between in vitro- and in vivo-derived neuronal datasets, while 535 glia were similar to the fetal sample, consistent with the neuron-specific accumulation of mCH. 536 This clustering based on bins also showed that differences in methylation between neurons 537 were not evenly distributed throughout the genome but show regional variability.

538



Martin et al., Supplementary Figure 7

539

540 Supplementary Figure 7 DNA methylation in CG context in gene bodies sorted for 541 differences in mCH between neuronal samples

Genes are in the same order based on CH methylation difference as in Fig 5B but showing
CG methylation for gene bodies and flanking 10 kb for fetal mouse frontal cortex (fetal), NeuNnegative cells from 7-week adult mouse prefrontal cortex (glia), 7-week adult mouse prefrontal
cortex neurons (in vivo neurons), and d38 mESC-derived neurons (in vitro neurons).
Difference in mCH between both neuronal samples used for gene order is shown on the right.



Martin et al., Supplementary Figure 8

548

549 Supplementary Figure 8 Cytosine coverage in gene bodies sorted for differences in 550 mCH between neuronal samples

Genes are in the same order based on CH methylation difference as in Fig 5B but showing average number of covered cytosines per bin for gene bodies and flanking 10 kb for fetal mouse frontal cortex (fetal), NeuN-negative cells from 7-week adult mouse prefrontal cortex (glia), 7-week adult mouse prefrontal cortex neurons (in vivo neurons), and d38 mESC-derived neurons (in vitro neurons). Difference in mCH between both neuronal samples used for gene order is shown on the right.

557

558 Finally, we generated ranked lists of genes based on either similarity or difference in genic 559 methylation between neuronal populations and performed gene set enrichment analysis 560 (GSEA) to examine correlation with biologically relevant pathways. We composed a pathway 561 package by combining gene ontology as well as reactome pathway datasets and performed 562 GSEA on both gene lists. Initially, we performed GSEA based on differences in methylation 563 levels (Figure 5E and 6E). For mCG, top pathways with higher gene body methylation in vitro 564 neuronal activity and synapse formation. Due to the generalised represented 565 hypermethylation in the CG context, relatively few pathways were enriched for genes with 566 lower methylation values (at p<0.05, Figure 5E), none of which directly related to neurons. Full 567 details of the top 50 hyper- and hypomethylated pathways for mCG context are listed in 568 Supplementary Figure 9. For mCH, top pathways enriched for genes hypermethylated in in 569 vitro neurons included morphogenesis and development, including brain and central nervous 570 system development. However, unlike mCG, differences in neuronal activity and synapse 571 formation were not detected. Genes hypomethylated in the mCH context in *in vitro* neurons 572 included pathways for cell cycle, cell division and DNA repair, but again, no pathways relating 573 directly to neurons were identified (Figure 6E). Full details for the top 50 of hyper- and 574 hypomethylated pathways for mCH context are listed in Supplementary Figure 10. To 575 simultaneously identify gene sets that share similar methylation states in both in vitro and in 576 vivo neuron populations and discriminate them against fetal or glial cells, we applied GSEA 577 on genes ranked by a combination of similarity between the mESC-derived in vitro neurons 578 and *in vivo* neurons, and dissimilarity to non-neuronal cell types (glia and fetal brain cells, 579 Figure 7). This analysis was performed for mCG and mCH independently and resulted in an 580 enrichment for pathways linked to genes that have an equivalent methylation

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| adult belavier 1,52 1,9e 5 Processive synthesis on the logging synth of cultur response cel projection morphogenesis 1,52 1,6e 5 regulation of cultur response central encoded 1,51 1,6e 5 Valeboardine assembly -1,61 1,8e central encoded 1,55 1,6e 5 Operation of cultur response -1,61 1,8e dwalert merganic catter transport 1,48 1,6e 5 Operation of new CENPA containing rubescatteres -1,61 1,8e out in development 1,48 1,0e 1,0e 5 Operation of new CENPA containing rubescatteres -1,61 1,8e out in development 1,44 1,0e 1,0e 5 Chornosame Mannesance -1,12 1,7e Axon guidate 1,44 1,0e 1,0e 5 Matexisting rubescatteres -1,12 1,7e Axon guidate 1,42 1,6e 35 SinRNP Assembly -1,72 7,7e 37e | cell morphogenesis involved in differentiation | | 1,53 | 1.0e-05 | Biosynthesis of maresin-like 5P Ms | 1 1 1 1 1 1 1 1 1 1 1 1 | -1,45 | 4.5e-02 |
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| | | 0 5660 10000 15000 20000 | 5 | | | 0 5000 10000 15000 20 | 1200 | |

581

582 Supplementary Figure 9 Top 50 enriched pathways for genes differentially methylated 583 in CG context between in vitro neurons and in vivo neurons

584 Pathways were ranked by enrichment score (NES), whereas positive NES indicates pathways

585 enriched in genes hypermethylated for CG in in vitro neurons, while negative NES indicates

586 pathways enriched in hypomethylated genes. Gene rank plots show position of genes being

587 part of a pathway set within the ordering of all genes based on methylation difference in mCG.

588 Pathways with p value larger than 0.05 shown in red.

| | | | | Martin et al., Supplementary Fig | ure 10 |
|--|--|------|------------|---|------------|
| Patrasy | Gene ranks | NES | (coak) | Pathway Generanks MI | 5 mai |
| paters specification process. | | 2.22 | 1.1e-05 | shcournein biosynthesic process | S 1.9e-04 |
| Xeobotics | MALIFICATION AND AND AND | 1.98 | 1.5e-05 | Cell Cycle Checkeoins | 6 1.4c-04 |
| Amine ligand-binding receptors | 1004 | 1.98 | 3.1e-05 | ncRNA metabolic process *********************************** | 7 8.7e-05 |
| embryopic morphogenesis | | 1.95 | 1.1e-05 | Resolution of 5 story Chromatial Cobesium | 8 2.1c-03 |
| embryonic organ development | | 1.94 | 1.1#-05 | regulation of symbolis encountersity mutualism through parasitism """ | 1 1.90-04 |
| Synthesis of epoxy EET and dihydroxycicosationoic acids DHET | **** | 1.94 | 8.20-05 | Cell Cycle 14 | 2 1.3e-04 |
| Synthesis of 16-20 -hydroxynicosatetraenoic acids HETE | 1 | 1.93 | 1.10-04 | abha aming acid metabolic process *********************************** | 2 5.3e-05 |
| Endosomal Vacuolar pathway | lava - | 1.92 | 1.30-04 | Post-franslational protein modification 111 | 4 7.28-05 |
| Fatty acids | 181811 8 | 1.91 | 1 50-04 | celular amino acid metabolic process | 5 6.54-05 |
| giand development | | 1.99 | 1.1e-05 | Cell Cycle, Mitotic 111 | 6 1.0e-04 |
| brain development | Inter set over 1 and 1 a | 1.88 | 1.1e-05 | Intra-Golgi and retrograde Golgi to-ER staffic | 6 5.4e-05 |
| ER-P hagosome pathway | 1000 | 1.88 | 4.4e 04 | Fatty acit, triacygrycero; and ketone body metabolism | 9 6.3c-04 |
| head development | | 1.87 | 1.1e-05 | protein glycosystem & matromolecule glycosystem | 0 1.0e-04 |
| Arachidonic acid metabolism | | 1.82 | 4.20-05 | DNA integrity direktorini | 1 3.5e-04 |
| central nervous system development | | 1.81 | 1.0e-05 | Mitotic Prometaphase | 1 5.50-05 |
| Cytochrome P450 - attanged by substrate type | | 1.81 | 1.1c-04 | Assembly of the primary clium 1 | 4.9e-05 |
| tube development | | 1.79 | 1.10-05 | shcosylation -1 | /4 5.3e-05 |
| Defensins | Increase in contract of the second | 1.78 | 7.80-04 | DNA Double-Strand Greak Repair | 75 4.7e-05 |
| Regulation of Beta-Cel Development | Banne and a second second second second | 1,78 | 1.6e-03 | Homology Directed Repair | 6 8.5e-05 |
| CYP 2E1 reactions | | 1.78 | 1.7e-03 | Cilum Assentiv | 0 5.3e-05 |
| embryn development ending in birth or egg hatching | his | 1.77 | 1.09-05 | DNA Read | 6 4.4e-05 |
| chordate embryonic development | | 1.77 | 1.0e-05 | N-stycan precursor dolchel lind-inkert olassaccharide, LLO and transfer to a nascert protein (1997) | 7 4.5e-04 |
| Prolactin receptor signaling | | 1.77 | 859-04 | Actuation of ATR in response to updication stress | /8 1.4e-03 |
| disassembly of the destruction complex and recruitment of AKIN to the membrane | 0.003 | 1.76 | 2.40-03 | restrosome onseization | 8 1.8e-04 |
| Phase 1 - Functionalization of compounds | | 1.74 | 1.2e-04 | Accuration N-linked abcossistion | 8 1.28-04 |
| Alpha-defensins | harve | 1.73 | 2.00-03 | DNA stand elogration | 40 1.7e-03 |
| WNT ligand biogeness and trafficking | 400 1111 - i ii | 1.71 | 4 40-03 | G2/M Checkpoints | 40 4.7e-05 |
| sceletal system development | In succession of the second se | 1.70 | 1.1e-05 | Organelle biogenesis and maintenance 1 (more series -1) | 1 5.7e-05 |
| reproductive system development | | 1.70 | 1.1e-05 | protein homosetramerization | 3 3.3e-04 |
| Antigon Presentation: Folding, assembly and people loading of class I MHC | | 1.70 | 3.60-03 | Base Excloin Repair | 3 1.1e-03 |
| reproductive structure development | han | 1.59 | 1.1e-05 | Mitote G2-G2 M phases 111 minutes -11 | 4 1.2e-04 |
| GPCR lizand binding | | 1.69 | 1.1e-05 | Post-tianslational modification: swithess of GPI-anchoved proteins | 5 1.3e-03 |
| sensory organ development | | 1.68 | 1.18-05 | spindle assentivy | 8 2.0e-04 |
| regulation of neuron differentiation | | 1.57 | 1.1e-05 | cellular amino acidi biosynthetic process | 8 1.7e-04 |
| Transcriptional regulation by the AP-2 (TEAP 2) family of transcription factors. | A Research and the second | 1.66 | 9.0e-03 | Resultion of cholesterol biosynthesis by SREEP SZEEF | 2 2.3e-04 |
| tissue morphovenesis | | 1.64 | 1.10-05 | Anchoring of the based body to the plasma membrane | 3 7.4e-05 |
| heart development | | 1.64 | 1.10-05 | G2 M baselon | 3 3.9e-05 |
| Nuclear signaling by EBBB 4 | Here's to a second to a g | 1.64 | 2.80-03 | Depdension-dependent mRNA decay management in management | M 1.8e-04 |
| newon projection morphogenesis | | 1.63 | 1.1e-05 | Regulation of FLK1 Activity at G2IM Transition | 6 7.3e-05 |
| Growth termone receptor signaling | | 1.52 | 6.42-03 | HDE through Homologius Recombination (HRR) | 0 6.7e-05 |
| Glacuropidation | 1 *** ****** | 1.52 | 1.3e-02 | HDR through Single Strand Ameaing (SSA) | 2 1.2e-04 |
| epithelial cell differentiation | | 1.60 | 1.14:05 | Recruitment of mitroic centrosame proteins and complexes | 4 3.60-05 |
| monohogenesis of an enithelium | | 1.60 | 1.1e-05 | Centrosome maturation | 4 3.5e-05 |
| cell motohogenesis involved in neuron differentiation | | 1.50 | 1.1e-05 | Anchoring of the basal body to the plasma memorane | 5 3.8e-05 |
| GABA receptor activation | Betteren a constraint | 1.59 | 7.10-03 | Loss of Np from mitoric centrosomes | 6 6.Se-05 |
| negative regulation of cell differentiation | | 1.58 | 1.10-05 | Loss of proteins required for interchase microholase organization from the centrosome | 6 6.9e-05 |
| regulation of neuropenesis | | 1.58 | 1.10-05 | AURKA Activation by TPX 2 | 9 6.9e-05 |
| suratic transmission | | 1.58 | 1.19-05 | protein A2P libosvistion -2 | 1 2.6e-05 |
| Class A/1 (Rhodoosin-like receptors) | | 1.58 | 6.9e-05 | Presynaptic phase of homologous DNA paring and sy and exchange | 6 2.9e-05 |
| Peptide ligand-binding receptors | | 1.57 | 7.6e-04 | Homologous DNA Paking and Strand Exchange | 8 3.0e-05 |
| | a som then there alone | 0200 | 1000000010 | a way take your peak | |

589

590 Supplementary Figure 10 Top 50 enriched pathways for genes differentially methylated 591 in CH context between in vitro neurons and in vivo neurons

592 Pathways were ranked by enrichment score (NES), whereas positive NES indicates pathways 593 enriched in genes hypermethylated for CH in in vitro neurons, while negative NES indicates 594 pathways enriched in hypomethylated genes. Gene rank plots shows the position of genes 595 being part of a pathway set within the ordering of all genes based on methylation difference in 596 mCH.

597

598 state in both of the neuronal samples. It is important to note that this analysis enriches for 599 similarity irrespective of overall methylation levels, whether high or low. When considering 600 mCG, the most represented pathways belonged to two major groups: neuronal function or cell 601 cycle. While neuronal terms are expected to be shared between neurons, the enrichment of 602 the latter group is likely due to its associated genes having a different methylation state in 603 post-mitotic neurons, compared to actively proliferating cells. Interestingly for mCH, with the

exception of Wnt signalling, the most highly enriched pathways did not relate directly to neurons, but included pathways related to chromatin organization, transcription and splicing (Figure 7 and Supplementary Figure 9). Together with the observed differential localisation of mCG and mCA in neuronal nuclei, this data suggests that rather than being directly involved in neuronal specification, mCH could play a role in the dynamic reorganisation of chromatin (Fraser et al., 2015) and the regulation of alternative splicing that occurs during neuronal maturation (Hubbard et al., 2013, Weyn-Vanhentenryck et al., 2018).



Martin et al., Figure 7

611

Figure 7 Enrichment for genes with similar methylation patterns in in vitro neurons and in vivo neurons

(A) Differences in gene body mCG level between neurons and fetal brain as well as glia (xaxis), and differences in gene body mCG level between both neuronal populations (y-axis), for all 24,049 genes with WGBS coverage. Dots are coloured by the similarity score that was used for gene set enrichment. Blue contour plot is based on gene density. (B) Selection of top enriched pathways from GSEA using similarity scoring from mCG information. (C) Differences in gene body mCH level between neurons and glia (x axis), and differences in gene body mCH level between both neuronal populations (y-axis), for all 24,049 genes with WGBS coverage.

- 621 (D) Selection of top enriched pathways from GSEA using similarity scoring from mCH
- 622 information. Full details for the top 20 similarity-enriched pathways are listed in Supplementary
- 623 Figure 11.

624

Martin et al., Supplementary Figure 11

| Pathway | Gene ranks | NES | pval |
|--|--|-----------|---------------|
| Chromatin organization | | 4.27 | 1.1e-05 |
| MCG Neuronal system | | 4.09 | 1.0e-05 |
| Cellular responses to stress | NA MARKATANA AND AND A AND AND AND AND AND AND AN | 4.07 | 1.1e-05 |
| Transmission across chemical synapses | | 4.05 | 1.1e-05 |
| Organelle biogenesis and maintenance | | 3.95 | 1.0e-05 |
| Cell cycle | | 3.93 | 1.0e-05 |
| M phase | THE REAL PROPERTY AND A RE | 3.84 | 1.1e-05 |
| Transcription | A REPORT OF A DESCRIPTION OF A DESCRIPTI | 3.68 | 1.1e-05 |
| Assembly of the primary cilium | THE CONTRACTOR CONTRAC | 3.58 | 1.1e-05 |
| Translation | THE PERSON PROPERTY AND A DESCRIPTION OF A DESCRIPTIONO OF A DESCRIPTION OF A | 3.51 | 1.1e-05 |
| Signaling by Wnt | A DECEMBER OF | 3.26 | 1.1e-05 |
| TCF dependent signaling in response to Wnt | | 3.21 | 1.1e-05 |
| Signaling by Rho GTPases | | 3.17 | 1.0e-05 |
| Mitotic anaphase | | 3.16 | 1.1e-05 |
| separation of sister chromatids | A CONTRACTOR DESIGNATION OF AN ADDRESS OF A DESIGNATION OF A DESIGNATION OF A DESIGNATION OF A DESIGNATION OF A | 3.06 | 1.1e-05 |
| Mus musculus biological processes | | 3.00 | 1.1e-05 |
| Axon guidance | | 2.97 | 1.0e-05 |
| Post-translational protein modification | | 2.97 | 1.0e-05 |
| Developmental biology | | 2.77 | 1.0e-05 |
| Signalling by NGF | 0 5000 10000 15000 20000 | 2.75 | 1.0e-05 |
| Pathway | Gene ranks | NES | pval |
| Translation | Research and the second s | 6.62 | 1.1e-05 |
| mRNA splicing | | 5.72 | 1.2e-05 |
| Transcription | ALMONDATION ALMOND ALMOND ALLONG ALL ALL ALL ALL ALL ALL ALL ALL ALL AL | 5.30 | 1.1e-05 |
| Citric acid TCA cycle and respiratory electron transport | | 4.89 | 1.2e-05 |
| Chromatin organization | ACCOUNTS AND A DESCRIPTION OF A DESCRIPR | 4.55 | 1.1e-05 |
| Cellular responses to stress | NOT WIND WIND WIND WIND WIND WIND WIND DISCUSSION OF A DAMAGE | 4.36 | 1.1e-05 |
| Organelle biogenesis and maintenance | BIRGE BIRG IN THE PARTY OF BELLEVILLE AND IN THE REAL PROPERTY OF THE RE | 3.93 | 1.1e-05 |
| Cellular senescence | | 3.64 | 1.2e-05 |
| Membrane trafficking | | 3.54 | 1.1e-05 |
| TCF dependent signaling in response to Wnt | CANADAR CONTRACTOR AND A LEAST DECIDE AND A LEAST CONTRACTOR AND A | 3.47 | 1.1e-05 |
| Signaling by Wnt | COMPANY NUMBER AND ADDRESS OF AN OTHER ADDRESS OF A DECOMPANY OF ADDRESS | 3.34 | 1.1e-05 |
| C-type lectin receptors CLRs | INTERNET STREET, AND AND AND A REPORT OF A DESCRIPTION OF A DESCRIPTION OF A DESCRIPTION OF A DESCRIPTION OF A D | 3.19 | 1.2e-05 |
| M phase | | 3.04 | 1.1e-05 |
| Cell cycle checkpoints | NUMBER OF BUILDING OF A CONTRACT OF BUILDING AND ADDRESS. | 2.99 | 1.2e-05 |
| Assembly of the primary cilium | THE REPORT OF THE TRANSPORT OF THE TRANSPORT OF THE DESIGN OF THE TAXABLE TO TAXABLE TO TAXABLE TO TAXABLE TO T | 2.96 | 1.1e-05 |
| Cell cycle | | 2.74 | 1.0e-05 |
| Programmed cell death | CONTRACTOR DE LA COMPANY DE LA CONTRACTOR DE LA CONTRACTOR DE LA COMPANY DE LA CONTRACTÓ DE LA CONTRACTÓ DE LA C | 2.74 | 1.1e-05 |
| S phase | NUMBER OF ADDRESS OF ADDRES | 2.69 | 1.2e-05 |
| Signaling by Hedgehog | | 2.68 | 1.2e-05 |
| | | 2001210-0 | 1090577000075 |

625

626 Supplementary Figure 11 Enriched pathways for genes with similar methylation 627 patterns for in vitro neurons and in vivo neurons

628 Pathways were ranked by enrichment score (NES), based on similarity between in vitro and

- 629 in vivo neurons and dissimilarity to glia and fetal brain in mCG and mCH context respectively.
- 630 Gene rank plots show the position of genes being part of a pathway set within the ranking of
- 631 all genes.

633 Discussion

634 The complex dynamics, composition, and patterns of DNA methylation observed in the 635 development and maturation of postnatal neurons is hypothesized to play an important role in 636 modifying gene expression and consolidating both neuronal cell types and their response to 637 activity (Cortes-Mendoza et al., 2013, Day et al., 2013, Feng and Fan, 2009, Graff et al., 2012, 638 Miller and Sweatt, 2007, Stroud et al., 2017). Mouse studies have identified some factors 639 involved in the process, including the DNA methyltransferases catalysing the deposition of mC 640 (Guo et al., 2014, Nguyen et al., 2007), and methylation readers such as MeCP2 that link DNA 641 methylation to gene expression (Chen et al., 2015, Fasolino and Zhou, 2017, Kinde et al., 642 2016, Lagger et al., 2017, Mellen et al., 2012, Skene et al., 2010, Stroud et al., 2017). 643 However, many basic questions remain unanswered, including the roles and regulation of 644 dynamic methylation events, and the factors that define the targeting of methylation sites. 645 Developing a robust *in vitro* model system to recapitulate the diverse methylation events 646 particular to neurons is important to facilitate the detailed molecular dissection of these 647 processes. In the present study we have extended an established protocol to differentiate 648 cortical neurons from mESCs and shown that these cells acquire in vivo levels of non-CG 649 methylation in a similar time frame to *in vivo* brain development. Furthermore, we have shown 650 that the timing of mCH deposition in vitro correlates to a transient increase in Dnmt3a 651 expression, which also recapitulates that observed in vivo (Lister et al., 2013). If, as these 652 results suggest, the deposition of mCH and *Dnmt3a* expression is indeed hardwired into the 653 developmental process, this has profound implications for studying the equivalent processes 654 in human iPSC-derived neurons. The human brain has a much more extended developmental 655 timeline compared to mouse (Stiles and Jernigan, 2010), and maximal in vivo mCH 656 methylation levels are not observed until late adolescence (16+ yr) (Lister et al., 2013). The 657 most advanced in vitro human cerebral organoid differentiation protocols currently available 658 can only recapitulate relatively early embryonic developmental stages (reviewed (Benito-659 Kwiecinski and Lancaster, 2019)). Whether further development of human cerebral organoids

through *in vitro* vascularisation (Cakir et al., 2019) or transplantation into the mouse (Mansour et al., 2018) can overcome this developmental obstacle and accelerate human neuron maturation to experimentally tractable time scales is currently unknown. To our knowledge, the mouse data presented here is the first report of mammalian neurons derived *in vitro* from pluripotent cells that harbor mCH at levels similar to those present in neurons *in vivo*, and opens the door to further, targeted investigations of the regulatory pathways and environmental factors involved.

667 Detailed analysis of DNA methylation levels and genomic distribution in mouse ESC-668 derived neurons identified several interesting features. Firstly, the levels and distribution of 669 mCG and mCH were regulated independently. Compared to the mouse brain neurons, 670 generalised hypermethylation in the mCG context, both within gene bodies and across the 671 genome, was not observed for mCH, which showed relatively normal gene body methylation 672 levels and slightly lower exon methylation. Regions of higher methylation in CG context were 673 not in general accompanied by higher methylation in CH context and vice versa, and regions 674 showing comparably lower methylation in one context did not show the same pattern in the 675 other context. These observations suggest that different regulatory mechanisms are involved 676 in the remodelling of neuron methylation patterns during maturation, depending upon the DNA 677 context and genomic feature targeted. As the mESC-derived neurons did not recapitulate 678 either of these methylation contexts with complete fidelity, it is likely that other factors, such 679 as environment, neuronal connectivity and activity, all act to develop the mature neuronal 680 methylome. Similarly, our comparisons to previously published iN cell data (Luo et al., 2019) 681 suggest that in this model system also, other factors are required to fully develop in vivo 682 methylation levels and patterns.

The early post-natal nuclear landscape of neurons is highly dynamic, with changes in gene transcription (Kang et al., 2011), alternative splicing (Furlanis and Scheiffele, 2018, Weyn-Vanhentenryck et al., 2018), DNA methylation (He and Ecker, 2015, Lister et al., 2013, Szulwach et al., 2011), and chromatin remodelling (Gallegos et al., 2018). It is likely that the

687 regulation of all these facets of neuron development are tightly interconnected. It is well-688 established that gene body mCH levels in neurons is inversely correlated with gene expression 689 (Chen et al., 2015, Chen et al., 2014, Gabel et al., 2015, Lister et al., 2013), and it has been 690 suggested that establishment of early post-natal mCH regulates the transcription of affected 691 genes at later time points (Stroud et al., 2017). Immunologically labelled mCA, both in vivo 692 and *in vitro*, shows a strong association with the nuclear periphery, suggesting association of 693 these genomic regions with the nuclear lamina. As the nuclear lamina forms a repressive 694 environment for transcription (Zuleger et al., 2011), association and dissociation of genes with 695 this environment can be a potent regulator of expression. During the neural induction of 696 mESCs, the pro-neural gene MASH1 is translocated away from the nuclear lamina, 697 concomitant with upregulation of its expression (Williams et al., 2006). Similarly, hundreds of 698 genes change lamina interactions during differentiation from mESC to neural progenitor cells. 699 and subsequently to astrocytes, and genes affected by altered lamina interactions reflect cell 700 identity and influence the likelihood of a gene being subsequently activated (Peric-Hupkes et 701 al., 2010). These findings suggest that lamina-genome interactions are centrally involved in 702 the control of gene expression programs during lineage commitment and terminal 703 differentiation. The association and dissociation of genes from the nuclear lamina is not 704 restricted to developing or differentiating cells. For example, the BDNF gene is translocated 705 away from the nuclear lamina, with a concomitant increase in expression, following stimulation 706 of mature neurons in vivo, proving that transcription-associated gene repositioning can occur 707 in adult neurons, as a result of enhanced activity (Walczak et al., 2013). While it is not yet 708 known how mCA associates with the nuclear lamina, or whether this is a direct association, 709 one possibility could involve binding to MeCP2 (Chen et al., 2015, Gabel et al., 2015, Lister et 710 al., 2013, Stroud et al., 2017). MeCP2 is a multifunctional protein, with reported roles in both 711 repression and upregulation of gene expression, as well as involvement in nuclear structure 712 (Chahrour and Zoghbi, 2007, Lagger et al., 2017, Young et al., 2005). In addition to binding 713 various methylated DNA species through its methyl-binding domain, including mCA, mCG, 714 and 5hmC (Chen et al., 2015, Gabel et al., 2015, Guo et al., 2014, Lagger et al., 2017), MeCP2

is able to interact directly with the lamin-B receptor (Guarda et al., 2009), a role that is independent of its function as an epigenetic reader protein. As levels of MeCP2 are very high in neurons, approaching that of H1 linker histone levels (Kishi and Macklis, 2004, Skene et al., 2010), it is tempting to speculate that one role of MeCP2 binding to mCA is to regulate its association with the nuclear lamina. The level of MeCP2 has been shown to increase during mESC-derived neuron differentiation (Yazdani et al., 2012) consistent with a close regulatory association to the increased levels of mCA observed here.

722 Generation of base resolution DNA methylomes of mESC-derived neurons allowed us 723 to compare pathways for which gene sets showed either the greatest similarity or the greatest 724 difference in methylation levels to in vivo neurons. Pathways hypermethylated in the mCH 725 context in ESC-derived neurons relative to in vivo neurons included a range of broad 726 developmental pathways, with no particular emphasis on neuron maturation or function. As 727 mCH has been shown to be deposited in the bodies of lowly expressed genes (Stroud et al., 728 2017), this suggests that these pathways have reduced functionality in post-mitotic ESC-729 derived neurons. In contrast, pathways with hypomethylation in the mCH context represented 730 the cell cycle and mitosis. These pathways are predicted to be silenced in post-mitotic cells, 731 suggesting that this hypomethylation occurs as a result of the inclusion of the represented 732 genes in tightly packed heterochromatin, although further studies are needed to confirm this. 733 Taken together, these data suggest that despite the different levels and labeling patterns for 734 mCH in the mESC-derived neurons, some fidelity is retained in the ESC-derived cells. 735 Interestingly, gene set enrichment pathways showing the greatest similarity in mCH between 736 in vivo and ESC-derived neurons include a range of regulatory pathways involved in 737 transcription, splicing and chromatin organisation, all aspects of neuron development that are 738 significantly modified in early postnatal neurons. Whether mCH is involved in the regulation of 739 alternative splicing or chromatin remodelling in neurons remains to be determined. However, 740 direct links between mCA and alternative splicing have been shown in human ESCs (Tan et 741 al., 2019) and CG methylation contributes to the inclusion or exclusion of alternatively spliced

exons in human cell lines (Maunakea et al., 2013). Furthermore, as MeCP2 can also be associated with alternative splicing and nuclear architecture (Yazdani et al., 2012, Young et al., 2005), a potential role for mCH in these processes should be considered.

745 The generalised CG hypermethylation in mESC-derived neurons challenged the 746 analysis of gene sets enriched for either similarity or difference to in vivo neurons, as the 747 highest ranked gene sets for both analyses represented different but closely related aspects 748 of neuron development and function. The mechanism underlying mCG hypermethylation is 749 not yet known. It could reflect either increased methylation, decreased activity of 750 demethylation pathways (Wu and Zhang, 2017), or an accumulation of 5hmC (Lister et al., 751 2013, Szulwach et al., 2011). or a combination of any or all three. The genome-wide increase 752 in mCG was observed to occur earlier in the neuron differentiation timeline than the maximal 753 increase in mCH, suggesting that these two processes are regulated independently.

Together, this work establishes that *in vitro* differentiation of mouse embryonic cells to neurons is a highly tractable and valuable model system with which to further dissect the roles of DNA methylation and higher order intranuclear architecture on neuron maturation and function.

758

759 Materials and Methods

760 Reagents and Antibodies

Primary antibodies are detailed in Supplementary Table 1. Directly-conjugated Alexa488mouse anti-NeuN was from Millipore (MAB377X). Mouse monoclonal antibodies to 5methylcytosine-adenosine dinucleoside (mCA) and 5-methylcytosine-guanine dinucleoside (mCG) were raised against KLH-conjugated dinucleosides at the Technology Development Laboratory (Babraham Bioscience Technologies Ltd, Cambridge, UK) (anti-mCA antibody clone 2C8H8A6 currently available from Active Motif, Cat. No 61783/4). Alexa Fluor secondary

antibodies were purchased from Life Technologies. ESGRO recombinant mouse Leukaemia
Inhibitory Factor (LIF) was obtained from Merck Millipore. The remaining reagents were
obtained from ThermoFisher Scientific or Sigma Aldrich unless otherwise specified.

770

771 Mouse Tissue Collection and in vitro neuron differentiation

Adult C57BL/6 mice (8-10 weeks old) were sacrificed and brains dissected and snap frozen on dry ice. Brains were then sectioned on a cryostat at 12 microns, placed on poly-L-lysine slides (VWR) and stored at -80°C until processing. All experimental procedures were approved by the Animal Welfare, Experimentation and Ethics Committee at the Babraham Institute and were performed under licenses by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act 1986.

778 For primary neurons, hippocampal or cortical neurons were cultured from embryonic 779 day 18 C57BL/6 mouse brains as described previously (Lanoue et al., 2017). All procedures 780 were conducted according to protocols and guidelines approved by the University of 781 Queensland Animal Ethics Committee. Isolated E18 neural progenitors were either frozen 782 directly for WGBS (E18 samples) or plated onto 0.1 mg/ml Poly L-Lysine / 8 µg/ml laminin-783 coated plates in Neurobasal containing 2% B27, 0.5 mM L-glutamine and 1% Pen-Strep and 784 maintained for 14 days. 14DIV neurons were either dissociated with Accutase and frozen as cell pellets for WGBS or washed in PBS and fixed in 2% PFA/PBS for ICC. 785

786

787 Murine embryonic stem cell culture and in vitro neuron differentiation

Two mESC cells lines, R1 (ATCC SCRC-1011) derived from a 129X1 x 129S1 male blastocyst (Nagy et al., 1993) and G4 derived from a 129S6/SvEvTac x C57BL/6Ncr male blastocyst (George et al., 2007), were maintained on gamma-irradiated mouse embryonic

fibroblasts in mESC medium (Dulbecco's Modified Eagle Medium (DMEM) (GIBCO), 15% Hyclone defined FBS (GE Healthcare), 10³U/ml ESGRO LIF (Millipore), 1x L-Glutamax, 1x sodium pyruvate, NEAA 1x (Invitrogen), 0.1 mM beta-mercaptoethanol). Cells were fed daily and split based on confluency. As differentiation of the G4 mESC line was slightly more robust this was used routinely, and data shown refers to G4 ESC-derived neurons unless otherwise stated.

797 Neural differentiation was initiated by dissociating the mESC colonies using Accutase 798 and excess feeder cells were removed from the cell suspensions by panning on gelatin-coated 799 plates. Dissociated cells were counted and transferred to ultra-low attachment cell culture 800 plates at a dilution of 220,000 cells/ml, in differentiation medium (Dulbecco's Modified Eagle 801 Medium (DMEM) (GIBCO), 10% Hyclone defined FBS (GE Healthcare), 1x L-Glutamax, 1x 802 sodium pyruvate, 1x NEAA (GIBCO), and 0.1 mM beta-mercaptoethanol). Neural induction 803 was continued for 8 days, medium was changed every two days, and supplemented with 5 804 µM retinoic acid for the final 4 days. Cell aggregates were dissociated using Accutase, single 805 cell selected through a 40 µm cell strainer, and plated onto 0.1 mg/ml poly-ornithine / 4 µg/ml 806 laminin-coated plates in N2 medium (DMEM/F12, 1x Glutamax 1x N2 supplement, 20 µg/ml 807 insulin, 50 µg/ml BSA) at a density of 50,000-100, 000 cells/cm². After 2 days, cells were 808 changed into N2B27 (Neurobasal, 1x N2 supplement, 1x B27 supplement, 1x Glutamax). 809 N2B27 was changed every 4 days for 12 days, following which 50% media changes were 810 performed for the remaining culture time.

High potassium-mediated depolarisation was performed as previously described (Martin et al., 2014). Briefly, cells were washed with low K+ buffer (15 mM HEPES, 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM D-glucose, 0.5 mM ascorbic acid, 0.1% BSA, pH 7.4), transferred to high K+ buffer for 5 min (formulation as the low K⁺ buffer but using 95 mM NaCl and 56 mM KCl), washed again in low K+ buffer and either harvested directly for RNA, immediately fixed for ICC, or chased in growth medium for the appropriate times prior to fixation or RNA extraction.

818

819 Human in vitro neuron differentiation

820 Human neurons were generated following two different approaches: either as adherent 821 monolayer cultures via a neural stem cell intermediate population (Reinhardt et al., 2013) or 822 as cerebral organoids (Lancaster and Knoblich, 2014). For adherent cultures, PSCs were 823 disaggregated mechanically and cultivated as embryoid bodies in DMEM/F12 and Neurobasal 824 1:1 mix, 1% Glutamax (all Life Technologies) with 1:200 N2 Supplement (R&D Systems), 825 1:100 B27 supplement without retinoic acid (Miltenvi Biotec) supplemented with 10 µM SB-826 431542, 1 µM dorsomorphin (both Selleckchem), 3 µM CHIR 99021 (Cayman Chemical 827 Company) and 0.5 µM PMA (Sigma) for 3 days on petri dishes. On day 4, SB-431542 and 828 dorsomorphin were removed and 150 µM ascorbic acid (Cayman Chemical Company) was 829 added to the media. On day 6, cells were plated onto Matrigel- (Corning) coated TC dishes to 830 allow attachment of neuroepithelial cell types. Over several passages, neural stem cells were 831 enriched resulting in pure populations. These neural stem cells could be differentiated by 832 removing the small molecules from the media and switching to B27 supplement with retinoic 833 acid, resulting in a mixed population of beta3-Tubulin positive neurons and GFAP-positive glia 834 within 4 weeks. For cerebral organoids, PSCs were dissociated into single cells and plated in 835 a ultra-low-attachment 96 well plate using 9,000 cells/ well in E8 media (Life Technologies), 836 with 50 µM Y-27632 (Selleckchem). After 5-6 days, embryoid bodies were transferred to ultra-837 low-attachment 6-well TC plates in neural induction media (DMEM/F12, 1% Glutamax, 1% 838 non-essential amino acids and 10 µg/ml heparin (Sigma). After another 4-5 days, organoids 839 were embedded into Cultrex (R&D Systems) matrix and cultivated under continuous agitation 840 on an orbital shaker in cerebral organoid media (DMEM/F12 and Neurobasal 1;1 mix, 1% 841 Glutamax, 0.5% non-essential amino acids, 1:200 N2 Supplement, 1:100 B27 supplement 842 (Miltenyi Biotec) and 1:40,000 Insulin (Sigma), with media changes every 3 days until a 843 maximum age of 9 months.

844

845 RT-qPCR

846 Total RNA was extracted using the Macherey-Nagel NucleoSpin RNA kit, including on 847 column digestion of DNA with RNase-free DNase according to manufacturer's specifications. 848 Concentration and 260/280 ratios were quantified using a NanoDrop 1000 spectrophotometer 849 before cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Primers were designed 850 to span exon-exon boundaries wherever possible (Supplementary Table 2). When this was 851 not possible, samples were excluded if genomic DNA contamination was more than 10-fold 852 over the cDNA concentration. Quantitative PCR (gPCR) reactions used SsoFast Evagreen 853 (Bio-Rad) with cDNA template according to manufacturer's instructions, using a C1000 854 Thermocycler (Bio-Rad) and CFX software. Results were analysed as described previously 855 (Livak and Schmittgen, 2001).

856

857 Immunocytochemistry

858 Protein ICC was performed as described previously (Martin et al., 2013) on cells fixed 859 in 4% paraformaldehyde or 2% paraformaldehyde (Electron Microscopy Sciences). For double 860 and triple immunocytochemistry using antibodies to methylated DNA, an alternative sequential 861 labelling method was used. Briefly, cells were fixed in 2% PFA/PBS for 10-30 min, 862 permeabilised for 1 h in 0.5% TX-100, then depleted of residual methylated RNA using RNase 863 A at 10 µg/ml for 30 min at 37°C in PBS. Cells were subsequently blocked in PBS/0.2% 864 BSA/0.2% cold-water fish skin gelatin for 10 min, then labelled with protein-targeting 865 antibodies in PBS/0.2% BSA/0.2% cold-water fish skin gelatin overnight at 4°C. Cells were 866 then washed with PBS, labelled using corresponding secondary antibodies in PBS/0.2% 867 BSA/0.2% cold-water fish skin gelatin for 30 min, washed in PBS, then re-fixed in 2% PFA/PBS for 15 min. DNA epitopes were retrieved using 4N HCI/0.1% TX-100 for 10 min at 868

room temp, cells washed in PBS/0.05% Tween-20, and blocked in PBS/0.05% Tween-20/1%
BSA (BS) for 1 h. Antibodies to methylated DNA were applied in PBS/0.05% Tween-20/1%
BSA overnight at 4°C. Cells were subsequently washed in PBS/0.05% Tween-20/1% BSA and
appropriate secondary antibodies applied in the same buffer containing 0.1 µg/ml DAPI for 30
min-1 h. Finally, cells were washed in PBS/0.05% Tween-20/1% BSA, rinsed in PBS and
either imaged directly (high throughput imaging), or mounted in Mowiol (Confocal).

875

876 Immunohistochemistry

877 Mouse brain sections were fixed in 2% PFA for 30 min, then permeabilised in 878 PBS/0.5% Triton X-100 for 1h, blocked in BS for 1h and incubated overnight at 4°C with a 879 NeuN antibody (1:2500) in BS. They were subsequently incubated for 1h with a secondary 880 fluorescently labelled antibody in BS (1:1000). After post-fixation with 2% PFA for 10 min, to 881 obtain access to DNA methyl groups, cell nuclei were mildly depurinised with 4N HCl treatment 882 for 15 min and incubated with antibody-enriched culture medium against mCG or mCA in BS 883 overnight at 4°C. After incubation for 1h with a secondary fluorescently labelled antibody in 884 BS (1:1000) the tissue was stained with 1:100 YOYO1 (Molecular Probes, Life Technologies) 885 for 15 min and mounted with Vectashield Antifade Mounting Medium (H-1000, Vector 886 Laboratories). Washing with PBS/0.05% Tween-20 for 1-2h was performed after each step, 887 reagents were dissolved in PBS/0.05% Tween-20 and steps performed at room temperature, 888 unless otherwise stated.

889

890 Microscopy

Phase contrast microscopy was performed on live cells using an Olympus IX51.
Confocal microscopy was performed on fixed cells using either a Zeiss 710 confocal
microscope and a 40x water immersion objective or a Leica SP8 confocal microscope using

a 60x oil immersion objective. High throughput imaging was performed using a Perkin-Elmer
Operetta equipped with a 20x Air objective. Brain section imaging was performed with Nikon
A1-R confocal microscope and a 60x 1.4 NA oil immersion objective.

897 Image analysis was performed by manual masking of nuclei and measuring 898 fluorescence intensity/nucleus using Image J (Figure 1J, L) or by high-throughput Operetta 899 image acquisition using Harmony (Figure 1D, F, H; Supplementary Figure 1E; Supplementary 900 Figure 2B), prior to exporting the images, generating nuclear masks and analysing 901 fluorescence intensity using Cell Profiler (Carpenter et al., 2006). All image analysis data was 902 collated using Excel 2016, and graphs prepared using Excel for Office 365 or Graphpad Prism 903 8. All images were processed using Adobe Photoshop 2020 and figures compiled with Adobe 904 Illustrator 2020.

905

906 Transmission electron microscopy

907 ESC-derived neurons were incubated with 10 µg/ml CTB-HRP in either high K⁺ or low 908 K⁺ buffer for 5 min, washed in PBS and fixed in 2.5% glutaraldehyde (Electron Microscopy 909 Sciences) for 24 h. Following fixation, cells were processed for 3, 39-diaminobenzidine (DAB) 910 cytochemistry using standard protocols. Fixed cells were contrasted with 1% osmium tetroxide 911 and 4% uranyl acetate prior to dehydration and embedding in LX-112 resin (Martin et al., 912 2013). Sections (~50 nm) were cut using an ultramicrotome (UC64; Leica). To determine CTB-913 HRP endocytosis, presynaptic regions were visualized at 60.000x using a transmission 914 electron microscope (model 1011; JEOL) equipped with a Morada cooled CCD camera and 915 the iTEM AnalySIS software.

916

917 Whole genome bisulfite sequencing

918 DNA methylation analysis by WGBS was performed using approximately 100.000 cells. 919 Genomic DNA was isolated with the DNeasy Blood and Tissue Kit (Qiagen) with some 920 modifications: samples were incubated for 4 h at 56°C with an additional RNAse A incubation 921 for 30 min at 37°C. 500 ng of genomic DNA spiked with 4% (w/w) unmethylated lambda phage 922 DNA (Thermo Fisher Scientific) was sheared to a mean length of 200 bp using the Covaris 923 S220 focused-ultrasonicator. Libraries for WGBS were prepared as follows: DNA fragments 924 were end-repaired using the End-It kit (Epicentre), A-tailed with Klenow exo- (NEB) and ligated 925 to methylated Illumina TruSeq adapters (BIOO Scientific) with DNA Ligase (NEB), followed by 926 bisulfite conversion using EZ DNA-methylation Gold kit (Zymo Research). Library fragments 927 were then subjected to 7 cycles of PCR amplification with KAPA HiFi Uracil+ DNA polymerase 928 (KAPA Biosystems). Single-end 100 bp sequencing was performed on a HiSeq 1500 or a 929 MiSeq (Illumina).

930

931 DNA methylation analysis

932 Reads were trimmed for guality and adapter sequences removed. Following pre-processing, 933 reads were aligned to mm10 or hg19 references with Bowtie and a pipeline described 934 previously (Langmead et al., 2009, Lister et al., 2009), resulting in a table summarising 935 methylated and unmethylated read counts for each covered cytosine position in the genome. 936 Bisulfite non-conversion frequency was calculated as the percentage of cytosine base calls at 937 reference cytosine positions in the unmethylated lambda control genome. This was performed 938 individually for each context (CA, CC, CG, CT). Methylation for particular genomic contexts 939 and average methylation of gene bodies were calculated by intersecting whole genome data 940 with feature bed files from the UCSC table browser using Bedtools (Quinlan, 2014). For 941 correlation, heatmap generation and clustering of methylation data, Deeptools was used 942 (Ramirez et al., 2016), along with R using packages pals, gplots, ggplot2, viridis and 943 RColorBrewer. When calculating average methylation level in a region (weighted methylation

944 level), the number of C basecalls divided by total sequence coverage at reference C positions 945 was used to calculate the methylation level for each context (CG, CH, CA, CC, CT). Sample-946 specific and context-specific bisulfite non-conversion rates, calculated from the unmethylated 947 lambda phage DNA control, were subtracted for each methylation context. Gene set 948 enrichment analysis was performed using the fasea package for R (Subramanian et al., 2005) 949 with reactome (Fabregat et al., 2018) and gene ontology annotations (Ashburner et al., 2000, 950 The Gene Ontology, 2019). Pathways were sorted by NES score (enrichment score 951 normalized to mean enrichment of random samples of the same size) and only pathways with 952 p-value < 0.05 were considered in subsequent analyses.

953 For similarity analysis, every gene was given a score as follows: First, the difference 954 in weighted DNA methylation between in vivo and in vitro neurons (y) was calculated for each 955 gene and scaled to a value between 0 and 1 (using the formula x = |y-1|), so that genes that 956 were more similar in methylation state between both samples would have a value (x) closer to 957 1. Then, the average of weighted DNA methylation per gene in neuronal samples was 958 compared against the average weighted DNA methylation of glial and fetal samples, resulting 959 again in a score between 0 and 1, with genes showing greater differences having a value 960 closer to 1. Both scores for similarity between neurons and dissimilarity to non-neuronal 961 samples were added in order to give both aspects the same weight, and scaled to values 962 between 0 and 1, with values closer to 1 representing genes being more similar in methylation 963 state between both neuronal samples but different compared to glia and fetal frontal cortex. 964 This similarity score was then used to rank all genes for GSEA using the fgsea package as 965 described above.

966

967 Fluorescence-activated nuclear sorting

968 Intact nuclei were isolated from cell pellets as described previously (Li et al., 2014,
969 Okada et al., 2011). Briefly, cells were Dounce-homogenised on ice in chilled nuclear

970 extraction buffer (10 mM Tris-HCl, pH 8, 0.32 M sucrose, 5 mM CaCl₂, 3 mM Mg(Ac)₂, 0.1 mM 971 EDTA, 1 mM DTT, 1x protease inhibitor cocktail (Merck), 0.3% Triton X-100). Nuclear lysates 972 were filtered (40 µm), centrifuged for 7 min at 3000 rpm at 4°C and resuspended in PBS. 973 Nuclei were blocked with 10% normal goat serum and labelled for 60 min on ice with either 974 directly conjugated mouse anti-NeuN-Alexa488, or pre-conjugated rabbit anti-Nanog/goat 975 anti-rabbit Alexa488 or rabbit anti-Pax6/goat anti-rabbit Alexa488 complexes. Samples of 976 each nuclear fraction were retained for secondary only antibody controls. 7-AAD (20 µg/ml) 977 was added all samples 15 min prior to sorting. A BD Influx cell sorter was used to sort nuclei. 978 Prior to sorting, the secondary only control was used to gate events to isolate nuclei from cell 979 debris. From the selected nuclear populations, nuclei were separated into distinct NeuN+ve/7-980 AAD+ve and NeuN-ve/7-AAD+ve populations, Nanog+ve/7-AAD+ve and Pax6+ve/7-AAD+ve 981 populations, depending upon the cell type.

982

983 Statistical Analysis

All data was analysed using an unpaired, two-tailed Student's t-test, unless stated otherwise.

985

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993

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1008

1009 Data availability

1010 WGBS data is available in GEO under the accession number GSE137098.

1011

1012

1013

1015 Table 1 Antibodies

1016

| Target | Host | Clone | Supplier | Catalog # |
|--------------------------|------------|-----------|--|-----------|
| TUBB3 (Beta III Tubulin) | Rabbit | | Sigma-Aldrich | T2200 |
| TUBB3 (Beta III Tubulin) | Chicken | | Merck-Millipore | AB9354 |
| NeuN (ICC) | Rabbit | D4G4O | Cell Signaling Technology | 24307 |
| NeuN (IHC) | Rabbit | | Abcam | ab128886 |
| NeuN-Alexa488 | Mouse | A60 | Merck-Millipore | MAB377X |
| c-Fos | Guinea pig | | Synaptic Systems | 226 004 |
| Synapsin 1 | Rabbit | | Merck-Millipore | AB154 |
| Pax6 | Rabbit | Poly19013 | BioLegend | 19013 |
| 5-mC | Rabbit | RM231 | Abcam | ab214727 |
| 5-hmC | Rabbit | | Active Motif | 39769 |
| mCA | Mouse | 2C8H8A6 | Available through Active Motif | 61783/4 |
| mCG | Mouse | 3A7 | Reik Laboratory, Babraham Institute | - |
| GFAP | Rabbit | | Dako | Z0344 |
| Nanog | Rabbit | D2A3 | Cell Signaling Technology | 8822 |

1017

1019 Table 2 RT-qPCR primers

1020

| Target | Gene ID | Forward primer | Reverse Primer | Ref. |
|-------------|------------|--------------------------|------------------------|-----------------------|
| beta-Actin | 11461 | AAGATCAAGATCATTGCTCCTCCT | CAGCTCAGTAACAGTCCGCC | - |
| NeuN/RbFox3 | 52897 | ATCGTAGAGGGACGGAAAATTGA | GTTCCCAGGCTTCTTATTGGTC | - |
| Dnmt1 | 13433 | AAGAATGGTGTTGTCTACCGAC | CATCCAGGTTGCTCCCCTTG | (Zeisel et al., 2013) |
| Dnmt3a | 13435 | GATGAGCCTGAGTATGAGGATGG | CAAGACACAATTCGGCCTGG | - |
| c-Fos | 14281 | CCTACTACCATTCCCCAGCC | CTGTCACCGTGGGGATAAAG | (Mikuni et al., 2013) |

1021

1022

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