

Characterization of DNA hydroxymethylation in the hypothalamus of elderly mice with post-operative cognitive dysfunction

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Abstract. Post-operative cognitive dysfunction (POCD) is a common syndrome characterized by perioperative cerebral damage in elderly patients, including cognitive impairment and memory loss. Recent studies have revealed that anesthesia is one of the key causes of POCD. Ubiquitin-like with PHD and ring finger domains 2 (Uhrf2) has been reported to play a crucial role in regulating DNA methylation and hydroxymethylation, which are closely connected with memory building and erasure. However, whether narcotic drugs can affect Uhrf2 to impact on DNA methylation and hydroxymethylation in POCD is poorly understood. In this study, a POCD model was established in elderly mice through sevoflurane treatment, and these mice were found to have compromised levels of global DNA 5'-hydroxymethylcytosine (5hmC) and Uhrf2 in the hippocampus and the amygdaloid nucleus, when compared with non-POCD and control mice. The results of immunoprecipitation and quantitative PCR revealed that 5hmC modification of the promoters of genes associated with neural protection and development, such as glial cell-derived neurotrophic factor, brain derived neurotrophic factor, glucocorticoid receptor and acyl-CoA synthetase short chain family member 2, was reduced in the hippocampus of POCD mice when compared with non-POCD and control mice. Taken together, the findings of the present study suggest that loss of 5hmC, in the hippocampus and the amygdaloid nucleus modulated by Uhrf2 suppression, may result in the learning and memory ability impairment seen in mice with POCD.

Introduction

Post-operative cognitive dysfunction (POCD) is a common syndrome in elderly patients, usually occurring in the first

several weeks or months after an operation. It results in dysfunction of the central nervous system, including cognitive impairment, declining learning and memory ability, information processing disorder and delirium (1). Clinical methodological differences between POCD studies, including the use of variable batteries of tests, lack of control groups, loss of patients during follow-up and inconsistent intervals between testing periods, limit their usefulness (2). Due to the lack of formal diagnostic criteria, as well as the subtlety of the cognitive changes that occur, assessment and diagnosis of POCD is difficult (3).

The causes of POCD in elderly patients are thought to be multifactorial and complex. One potential risk factor for POCD is the use of anesthetic agents (4). Widely used narcotics are currently classified into inhalational and intravenous anesthetics (5). Inhalational general anesthetics such as isoflurane and halothane have been demonstrated to increase the risk of Alzheimer's disease (AD) in the aging brain (6), and exert a neurotoxic effect via caspase-mediated apoptosis (7).

One proposed hypothesis is that epigenetic regulation influenced by anesthetic may be a critical mechanism underlying POCD (8). Despite the similar pathological change in neurocytes with AD, the epigenetic alteration in POCD predominantly results in memory and learning disabilities (9). DNA hydroxymethylation is a novel modification based on DNA methylation catalyzed by dioxygenases. Hydroxymethylated cytosine (5hmC) is identified as an intermediate of the active demethylation process (10,11). 5hmC is highly distributed in the early embryo, embryonic stem cells (12) and the nervous system (13,14). Recent studies have revealed that the level of 5hmC is reduced by 10% after ten-eleven translocation methylcytosine dioxygenase 1 (TET1) knockdown, which can retard the proliferation of neural progenitor cells and impair the abilities of spatial learning and memory (15,16). Moreover, a reduction in 5hmC modulates transcriptional activity of some genes involved in neurogenesis in AD mice, which also indicates that 5hmC is closely connected with memory maintenance (17,18). Along with the TET family, ubiquitin-like with PHD and ring finger domains 2 (Uhrf2) is also considered to be a novel regulator of 5hmC via its SET and ring finger associated domain (19,20). Aberrant distribution of 5hmC may be one of the possible molecular causes of POCD occurrence. The major enzymes that contribute to 5hmC metabolism affected by anesthetic in POCD are also poorly understood.

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In this study DNA hydroxymethylation in the central neural system of POCD mice was profiled in an attempt to reveal the underlying pathogenesis of POCD caused by anesthetic.

Materials and methods

Animal study. All procedures were approved by the Institutional Animal Care and Use Committee of Fudan University, Jinshan Hospital (Animal protocol number 2017-32-166). A total of 170 18-month-old outbred female C57BL/6 mice, purchased from Shanghai SLAC Laboratory Animal Co., Ltd., were used in this study. The levels of glucose and oxygen saturation (SpO₂) in 50 μ l blood isolated from the caudal vein were determined using a biochemical analyzer (Beckman Coulter Inc.). IL-1 β levels were analyzed in a further 50 μ l blood using ELISA kits (cat. no. PMLB00C; R&D Systems Inc.). Animals were fed with standard food and water *ad libitum*. A total of 150 randomly selected mice were treated with 2% sevoflurane for 2 h (21-23) in an anesthesia chamber with a size of 25x13x13 cm. The remaining 20 mice were treated with normal air, as a negative control. In a pilot study 2% sevoflurane treatment was tested for 4 h, but >50% of mice died upon this condition (data not shown).

The Morris water maze test (post-treatment 2-7 days) and open field test (post-operative day 7) were performed as previously described (24,25) in order to verify the POCD model. In brief, for the Morris water maze test, a platform was set in the center of a 0.5 m high and 1.2 m in diameter pool, 5 cm above the water surface. Mice were trained at the same entrance for a total of 2 min each time for a total of 6 times from the 2nd to 7th day after sevoflurane treatment (one exposure). The motion trail, the escape period and the distance travelled were recorded. For the open field test, mice were put in a 30x72x72 cm box, with light conditions that mimicked daytime. During the 5 min the mice spent in the box, their average distance travelled, total grid crossings, distance travelled around the central region and duration spent in the central region were recorded. Both an escape period of <90 sec after the 7th day of sevoflurane treatment and an open field score of <25% were considered as cognitive damage consistent with a POCD model (26,27).

The mice were sacrificed on day 7 by cervical dislocation. For each mouse, all brain tissues including hippocampus, amygdaloid nucleus and cerebellum were harvested and separated into two parts. One was fixed by 1% paraformaldehyde at room temperature \geq 24 h for DNA dot blot assay, Methylated DNA immunoprecipitation (MeDIP) and immunofluorescence assay, while the other is washed by cold PBS and treated with RIPA buffer (Solarbio Inc.) or TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) accordingly for western blotting and PCR.

DNA dot blot assay. A total of 2 μ l genomic DNA was extracted from tissues (Qiagen GmbH) and was dropped onto each nitrocellulose membrane at a 2-fold serial dilution (0, 5, 10, 20 and 40 ng) for dot blot assay. The spots were dried at room temperature and incubated in TBST with antibodies against 5hmC (cat. no. ab214728; Abcam) or 5-methylcytosine (5mC; cat. no. ab10805; Abcam) (1:500 dilution; 1 ng/ml) in 10 ml of TBS-T for 4 h overnight with gentle shaking. The membranes

were washed with TBS-T three times for 10 min each time at room temperature, followed by goat anti-mouse IgG-HRP (1:10,000 dilution; 20 ng/ml; A0216, Beyotime Institute of Biotechnology) or goat anti-rabbit IgG-HRP (1:10,000 dilution; 20 ng/ml; A0208, Beyotime Institute of Biotechnology) incubation in 10 ml of TBST for 1 h at room temperature with gentle shaking, and washed again three times. BSA (New England Biolabs Inc.) was used as a negative control. Membranes were subsequently incubated with 3 ml of ECL Western Blotting Substrate (Beyotime Institute of Biotechnology) for 5 min in darkness at room temperature to develop the bands by Tanon 5200 Chemiluminescence Imaging Analysis System.

Western blot analysis. Brain tissues were homogenized in RIPA buffer and then centrifuged at 4°C and 12,000 \times g for 10 min. The protein quantity in the supernatant was determined using a BCA protein assay kit (E162-01; Fansbio). Equal amounts of total 40 μ g protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were then blocked using 5% non-fat milk in TBS at 4°C for 90 min and then incubated with the respective primary antibodies against TET1 (1:2,000; cat. no. ab191698; Abcam), TET2 (1:2,000; cat. no. ab124297; Abcam), Uhrf1 (1:2,000; cat. no. 12387; Cell Signaling Technology, Inc.), Uhrf2 (1:2,000; cat. no. ab28673; Abcam) and β -actin (1:5,000; cat. no. AA128; Beyotime Institute of Biotechnology) overnight at 4°C. Membranes were washed with TBST and incubated with goat anti-mouse and goat anti-rabbit IgG-HRP (1:10,000; 20 ng/ml, Beyotime Institute of Biotechnology) at room temperature for 1 h. Membranes were then treated with aBM Chemiluminescence Western Blotting Kit (cat. no. 11520709001; EMD Millipore), and the bands were captured to evaluate the difference of protein expression.

Immunofluorescence assay. In brief, mouse tissue was fixed using 4% paraformaldehyde at room temperature for 24 h then dehydrated with 50-90% ethanol. Samples were permeabilized in 50% xylene-ethanol at room temperature for 30 min then in 50% xylene-paraffin at 60°C for 15 min and embedded. Tissues were cut into 5 μ m sections and washed with 0.3% H₂O₂-methanol for 10 min then heated to 92°C for 40 min for antigen retrieval. Following PBS washing, sections were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Subsequently, slices were incubated with 5hmC (1:200; cat. no. ab214728; Abcam) and Uhrf2 (1:200; cat. no. ab28673; Abcam) antibodies overnight at 4°C. After washing, tissues were further incubated with the appropriate Alexa Fluor secondary antibody (cat. no. A32732; Thermo Fisher Scientific, Inc.) at 1:20,000 dilution for 30 min at room temperature. After washing, cells were mounted in mounting media with DAPI (Vector Laboratories Inc.). The positive staining of 5hmC and Uhrf2 were digitally captured by Olympus BX51 (Olympus Corporation) at x400 magnification and analyzed using ImageJ software v1.8.0 (National Institutes of Health).

MeDIP quantitative (q) PCR assay. MeDIP assay was performed as previously described (28). Extracted genomic

Table I. Sequences of primers used.

A, Med IP assay		
Target	Primer sequence (5'-3')	Length of PCR products
ACSS2	CCCAGACCATAACAGTACCGACTC CGCCTTTGCCATTCATAGAGC	294
BDNF	TTAGAGGAGGTGTAGCCTTGTT TTGTCATCACAGTGGGAAGC	223
CCL2	AAAGTTTCCATTGCTGCTGCTC TCTGATGTAACGGGCTCTTGG	106
FAS	ACTTCCCTACCCACCCATTC AAAGTACCCAAGGAGCTAAAGG	220
GCR	ATTGCCCTGGATGCCTGTAA ATGACCATGAACCTCCTGAA	321
GDNF	ATGGCTCTATGCTGCTTTGC TATCCCAGACGTGGACTTGC	163
GLUR2	GAGGGTGGAATGGGAAAGAG AGGCAGCTACCAAATGTCTCG	197
HMGB1	TTTTCTTCTTTGGGTCTAA CCAGCCTAACTCTGCTTCCT	217
MMP9	TGGTTTCAGAAGAGGAGGACAGG GCAGCGAGGAACAGGGAGCA	248
B, qPCR assay		
Target	Primer sequence (5'-3')	Length of PCR products
ACSS2	TCCCATTCTTCGGTGTAGCG GTAACAAAGCAGTAGAGGCATTCG	376
BDNF	GTGGGTACACAGCGGCAGATA ACGATTGGGTAGTTCGGCATT	203
CCL2	AAGAAGGAATGGGTCCAGACA GCTTCAGATTTACGGGTCAACT	140
FAS	TCTGGGCTGTCCTGCCTCTG CAGTTTCACGAACCCGCCTC	111
GCR	TGGAATAGGTGCCAAGGGTC GCAGAGTTTGGGAGGTGGTC	171
GDNF	GATGAAGTTATGGGATGTCGTGG TGCCGCTTGTTTATCTGGTGA	175
GLUR2	GAAGCCTCAGAAGTCCAAACC TTAGCCGTGTAGGAGGAGATG	342
HMGB1	ATCCTGGCTTATCCATTGGTG TCCTCATCCTCTTCATCCTCCT	244
MMP9	AAGGGTACAGCCTGTTCTGCTG GATGCCGTCTATGTCGTCTTTATTCA	146
MedIP, methylated DNA immunoprecipitation; ACSS2, acyl-CoA synthetase short chain family member 2; BDNF, brain derived neurotrophic factor; CCL2, C-C motif chemokine ligand 2; FAS, fas cell surface death receptor; GCR, glucocorticoid receptor; GDNF, glial cell-derived neurotrophic factor; GluR2, glutamate receptor 2 precursor; HMGB1, high mobility group protein B1; q, quantitative; MMP9, matrixmetal-lopeptidase-9.		

DNA was sonicated (90 cycles of 30 sec on/30 sec off with high power; UCD-300; Bioruptor) and incubated with 0.5 μ g 5hmC (cat. no. ab214728; Abcam) or 5mC (cat. no. ab10805; Abcam) antibody or IgG (cat. no. A0208, Beyotime Institute

Table II. Principle indexes of an open field test.

Group	Sample size	Weight (g)	Blood glucose (mmol/l)	SpO2 (%)	IL-1 β (pg/m)	Average distance (cm)	Total across grids	Distance among central region (cm)	Staying time among central region (s)
Control	20	23.5 \pm 3.2	9.4 \pm 2.1	96.13 \pm 1.36	14.87 \pm 2.09	13.389 \pm 3.8	84 \pm 12	1.895 \pm 0.44	2.926 \pm 0.89
Non-POCD	132	23.7 \pm 1.8	10.1 \pm 3.3	95.87 \pm 2.14	15.39 \pm 1.56	12.354 \pm 4.9	81 \pm 20	1.754 \pm 0.33	3.179 \pm 1.04
POCD	18	23.8 \pm 2.5	9.8 \pm 2.8	95.61 \pm 1.08	15.14 \pm 1.91	8.94 \pm 2.2 ^{a,b}	54 \pm 14 ^{a,b}	1.246 \pm 0.38 ^{a,b}	2.67 \pm 2.44 ^{a,b}

^aP<0.05 vs. control and ^bP<0.05 vs. non-POCD groups. POCD, Post-operative cognitive dysfunction; IL-1 β , interleukin 1 β .

of Biotechnology) as control overnight to capture the DNA fragment with 5hmC or 5mC, then washed and harvested for detection of the 5hmC or 5mC enrichment at promoter regions of candidate genes, where the primers for qPCR were designed to encompass ~200 bp (Table I). The qPCR reactions were done using the Fast Universal SYBR Green Realtime PCR Master Mix (Roche Diagnostics) and in triplicate under the following conditions: 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Ct value was analyzed to calculate enrichment using the $2^{-\Delta\Delta C_q}$ method (29).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted using TRIzol[®] reagent according to the manufacturer's instructions. Before performing RT, RNA was treated with 5U DNase I (Beyotime Institute of Biotechnology) on ice for 10 min to remove bacterial genomic DNA, and purified using isopropanol and 3M sodium acetate, before washing with 75% ice ethanol. Reverse transcription was performed as the conditions of 42°C for 15 min and 95°C for 3 min using the QuantiTect Reverse Transcription kit (Qiagen GmbH). qPCR were performed using Fast Universal SYBR Green Realtime PCR Master (Roche Diagnostics) with the conditions followed were 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 30 sec using the primers listed in Table I.

Statistical analysis. To distinguish between the non-POCD and POCD groups, the escape latency values of control group at post-operative day 7 were collected and fitted a normal distribution as $\mu \pm \sigma$. Normal samples were distributed within the range from $\mu - 3\sigma$ to $\mu + 3\sigma$ by 99.73% probability. Thus, the values included or excluded in $\mu \pm 3\sigma$ were considered as non-POCD or POCD respectively. Likewise, the values of distance travelled as well as the data of open field test including average distance, grid crossings numbers, distance among central region and staying time among central region were all analyzed in the same way. Finally, the intersection of the potential POCD from each type of parameters was confirmed as the POCD group. Data are presented as the mean \pm standard deviation for multiple independent experiments. The multiple comparisons of the difference between values were analyzed using one-way ANOVA. The pairwise comparisons of control group and POCD/non-POCD groups were analyzed using Fisher's Least Significant Difference post hoc test following ANOVA. Pearson's correlation analysis was used to evaluate the association between 5mC and 5hmC and the mRNA level. P<0.05 was considered to indicate a statistically significant difference.

Results

5hmC expression is altered in mice with cognitive dysfunction. Initial levels of blood glucose, SpO2 and IL-1 β were analyzed in order to rule out respiratory depression, infection or hypoglycemia, because these physical signs were subject to POCD occurrence (Table II). A total of 150 mice were treated with 2% sevoflurane in order to establish a POCD model. A total of 18 of these mice were identified as having POCD via both Morris water maze test and an open field test daily between 2 and 7 days post-operatively (Fig. 1A; Table II). Unlike POCD in humans, which happens most conspicuously on post-operative

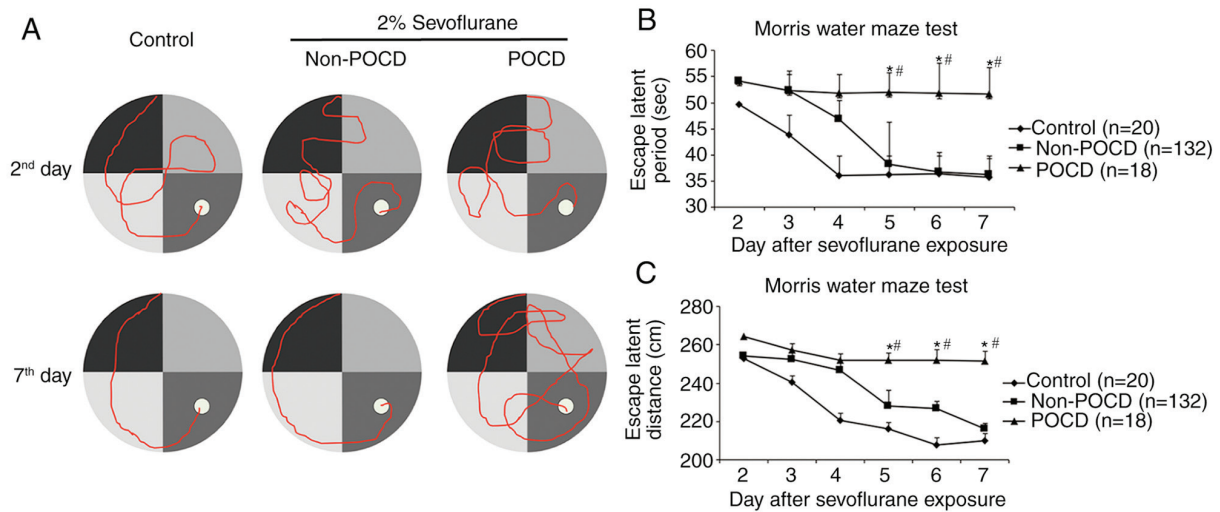


Figure 1. Verification of a POCD mouse model using Morris water maze test. (A) The movement route. (B) Escape latency period. (C) Distance travelled by mice in the control, non-POCD and POCD groups. All data are presented as the mean \pm standard deviation of each group. * $P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. non-POCD groups. POCD, post-operative cognitive dysfunction.

day 7 and at post-operative month 3 (30), POCD mice started to display increasing escape latency and travelled distance from the 5th day when compared to control and non-POCD mice (Fig. 1B and C). The mice were sacrificed at day 7 and the brain tissues harvested to detect global 5hmC expression via dot blot assay. The presence of differential change of global 5hmC expression in whole brain lysate before and after POCD was observed ($n=3$; Fig. 2A and B). Furthermore, the hippocampus, amygdaloid nucleus and cerebellum were also harvested on day 7 and it was observed that 5hmC was significantly lower in POCD mice compared to control in the hippocampus and amygdaloid nucleus, but no obvious change was seen in the cerebellum ($n=5$; Fig. 2C and D). There were no differences in 5mC level between POCD and control ($n=5$; Fig. 2E and F). Taken together, the loss of 5hmC in both the hippocampus and amygdaloid nucleus was observed in POCD mice and it may be responsible for the cognitive impairment seen, including the loss of abilities of memory, spatial learning, and new environmental adaptation.

Loss of Uhrf2 is responsible for 5hmC alteration in hippocampus of POCD mice. To elucidate the hydroxymethylation impacted by POCD, the enzymes Uhrf1, Uhrf2, TET1 and TET2 for 5hmC were investigated via western blotting. The protein levels of Uhrf1, TET1 and TET2 exhibited no significant differences between control and POCD in whole brain, whilst Uhrf2 displayed a slight downregulation in POCD ($n=5$; Fig. 3A). When considering the background noise from the whole brain, Uhrf2 was further investigated in specific regions of brain and observed a suppression in hippocampus and amygdaloid nucleus in POCD compared with control ($n=5$; Fig. 3B and C), coinciding with the change of 5hmC. Collectively, Uhrf2 was suppressed by sevoflurane for hindrance of 5hmC in POCD.

POCD results in 5hmC reduction at the promoter regions of genes associated with neurodevelopment. The present study hypothesized that Uhrf2 may be responsible for 5hmC maintenance in hippocampus and amygdaloid nucleus. To further

validate the role of 5hmC in POCD, the local 5mC and 5hmC enrichment at promoter regions of genes associated with neurodevelopment was detected through MedIP-qPCR. Glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), glucocorticoid receptor (GCR) and acyl-CoA synthetase short chain family member 2 (ACSS2) displayed a reduced 5hmC (Fig. 4A) and unvaried 5mC (Fig. 4B) level on their promoters in POCD compared with control (control $n=5$; POCD $n=6$). Compared with their transcriptional levels (Fig. 4C), 5hmC levels on the promoters of GDNF, BDNF, GCR and ACSS2 could reflect the transcriptional activation of these genes (Figs. 4D and S1). While 5mC levels on the promoters of MMP9, HGMB1 and GLUR2 showed an inverse tendency with transcription of these genes (Fig. 4B and D). Additionally, neither 5hmC nor 5mC of FAS and CCL2 had any correlation with their mRNA change in POCD compared to control.

Discussion

In the present study, 2% sevoflurane was used to establish a POCD model. Mouse models of POCD displayed signs of behavioral and memory problems (27,31), as indicated by the results of water maze and open field tests. By contrast, the results for the non-POCD group were similar to the normal control. Although certain phenotypes have been studied to classify early POCD, such as serum proteomics (32), cerebrospinal fluid (33), and cerebral oxygen saturation (34), no clear test for the susceptibility of individuals to POCD is available.

Due to a lack of potent drug for the treatment of POCD in clinical practice, a previous study has prompted the use of DNA methyltransferase inhibitors, which can restore memory-associated transcriptional regulation and improve behavioral memory function in elderly animals (8). However, the role of epigenetic factors in POCD has not been previously investigated to the best of our knowledge. The current study demonstrated an association between 5hmC and POCD, and aimed to clarify the role of hydroxymethylation in POCD for the regulation of memory and learning ability. The global 5hmC

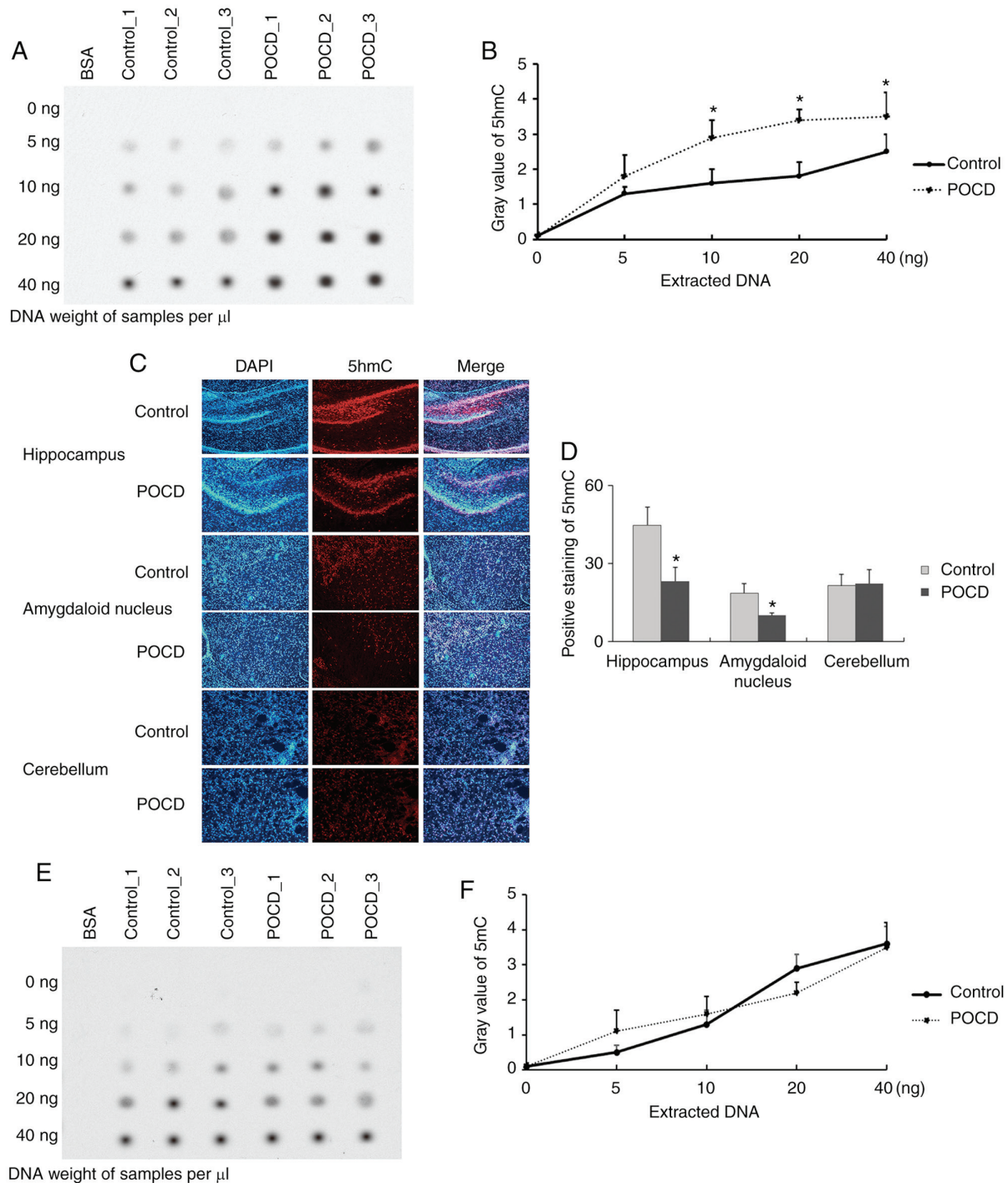


Figure 2. Characterization of 5hmC level in the brain of POCD mice. The global 5hmC level of whole brain using (A) dot blot assay and (B) statistical analysis. The 5hmC distribution in hippocampus, amygdaloid nucleus and cerebellum using (C) immunofluorescence and (D) statistical analysis. The global 5mC level of whole brain using (E) dot blot assay and (F) statistical analysis. Gray value was defined as the mean of the integrated optical density of the pixels in the selection. All data are presented as the mean \pm standard deviation of the mean of five independent experiments. * $P < 0.05$ vs. control group. 5hmC, 5-hydroxymethylcytosine; BSA, bovine serum albumin; POCD, post-operative cognitive dysfunction.

level in brain was reduced in POCD. However, of the different brain regions, 5hmC levels in hippocampus and amygdaloid nucleus declined the most markedly in the POCD group, which implies that 5hmC may contribute to memory and learning ability in the hippocampus (35) as well as fear emotion control in the amygdaloid nucleus (36). However, the presence of unaltered 5hmC levels in the cerebellum before and after POCD, suggests that the basic associative learning and memory from

cerebellum impaired by POCD (37) may be independent of accumulation or loss of hydroxymethylation (37). The expression levels of hydroxymethylation associated enzymes such as Uhrf1 and TETs remained unchanged, while the decreased protein levels of Uhrf2 in POCD were consistent with 5hmC levels both in the hippocampus and the amygdaloid nucleus. A previous study reported that TET1 knockout did not affect overall brain morphology in mice, and concluded that TET1

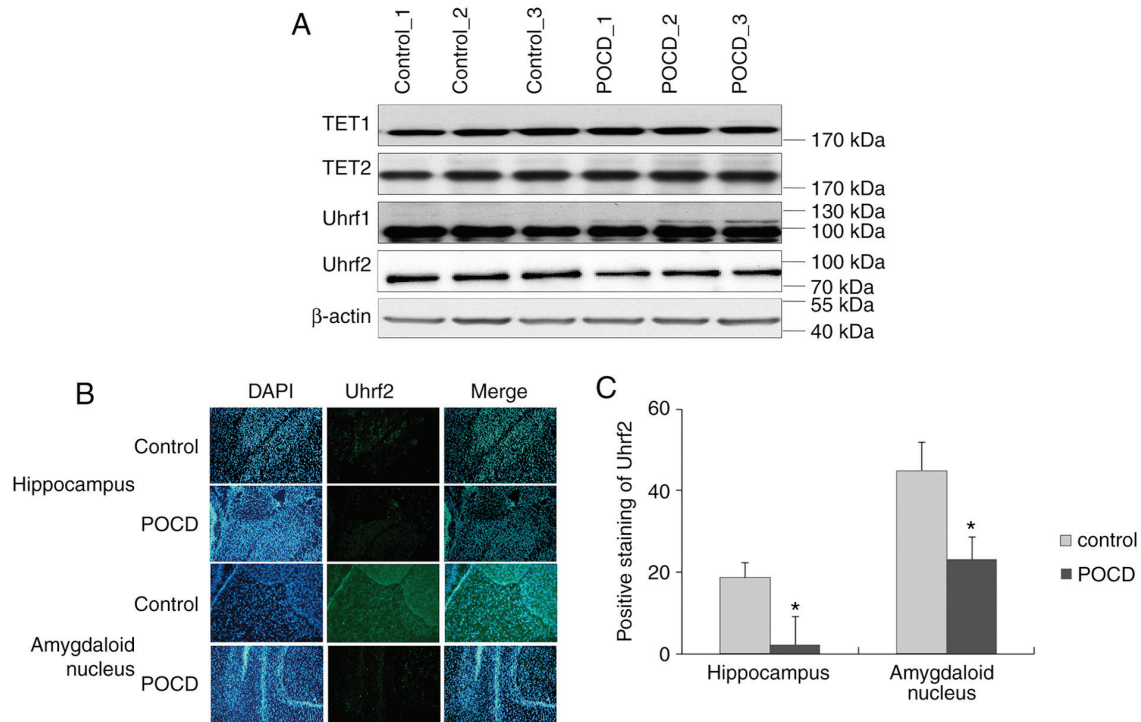


Figure 3. Expression of Uhrf2 in the brain of POCD mice. (A) The protein expression levels of enzymes associated with hydroxymethylation were evaluated in the whole brain using western blotting. The Uhrf2 protein distribution in hippocampus and amygdaloid nucleus using (B) immunofluorescence and (C) statistical analysis. All data are presented as the mean \pm standard deviation of the mean of five individual experiments. * $P < 0.05$ vs. control group. POCD, post-operative cognitive dysfunction; TET 1/2, ten-eleven translocation methylcytosine dioxygenase 1/2; Uhrf 2, ubiquitin like with PHD and ring finger domains 2.

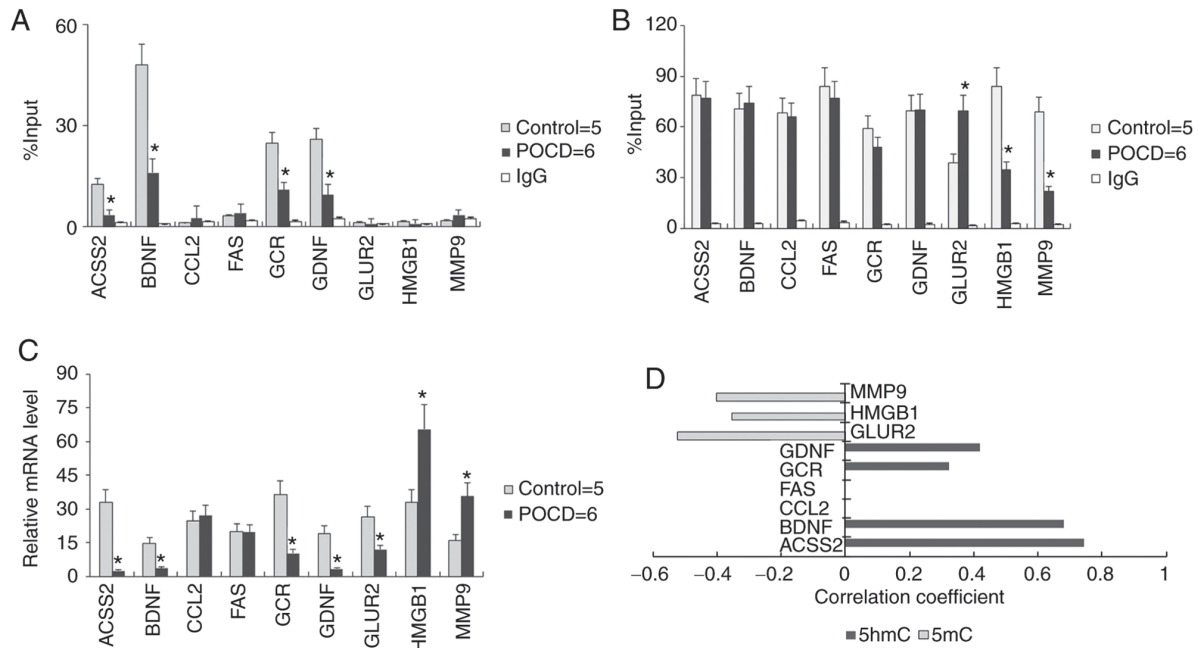


Figure 4. Association between 5hmC and gene transcription. Local (A) 5hmC and (B) 5mC enrichment at promoter regions of genes associated with neuro-development. (C) Transcriptional level of nine genes in mouse brain. (D) The significant difference $P < 0.05$ between 5mC or 5hmC enrichment and mRNA level are listed. All data are presented as the mean \pm standard deviation of the mean of individual experiments (control for five; POCD for six duplications). * $P < 0.05$ vs. control group. 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ACSS2, acyl-CoA synthetase short chain family member 2; BDNF, brain derived neurotrophic factor; CCL2, C-C motif chemokine ligand 2; FAS, fas cell surface death receptor; GCR, glucocorticoid receptor; GDNF, glial cell-derived neurotrophic factor; GluR2, glutamate receptor 2 precursor; HMGB1, high mobility group protein B1; MMP9, matrixmetalloproteinase-9.

deletion could enhance the consolidation and storage of threat recognition (cued and contextual fear conditioning) and object

location memories (38), which is apparently reciprocal with the phenotype of POCD. It is therefore unlikely that TET

levels exert an effect in POCD. A recent study reported that loss of Uhrf2 reduced 5hmC in the brain, including the cortex and hippocampus, but did not change 5mC level, and that mice exhibited a partial impairment in spatial memory acquisition and retention (20), which was further confirmed by the current results. The results of the present study suggest that Uhrf2 may be a target responding to 5hmC regulation in POCD.

The relationship between 5hmC enrichment and gene transcription in POCD was further investigated. Two epigenetic means of gene transcriptional regulation closely associated with neurodevelopment, DNA methylation-mediated gene silencing and loss of DNA hydroxymethylation-mediated gene silencing were identified in the current study. 5hmC was reduced in ACSS2, BDNF, GCR and GDNF, while 5mC levels for these genes remained unaltered, which indicated that sevoflurane can suppress Uhrf2 to compromise the 5hmC modification on the promoter regions of these genes associated with neuroprotection and proliferation and thereby repress the transcriptional activity. Moreover, the change of expression of GLUR2, HMGB1 and MMP9 were significantly negative correlated with their DNA methylation change at promoter level. This may be due to a change in DNA methylation patterns in their promoters.

The molecular mechanism underlying the differences between non-POCD and POCD remains to be elucidated. We hypothesized that it depends on individual factors, such as neuroplasticity, immunity and the sensitivity to sevoflurane. However, it is difficult to distinguish differences in the epigenetic patterns of brain tissues from a group of animals as variations exist between each individual. Once POCD develops, samples of the diseased brain in its pre-diseased normal state cannot be obtained. A further limitation is that incidence of POCD in both humans and mouse models is low. In addition, sevoflurane treatment may not be an optimal model for POCD induction. In future studies a rapid method for delirium induction by scopolamine (39) or another sedative may be a better way to mimic and further study POCD. An additional limitation of this study is that genetic editing (over-expression or knockdown *in vivo*) operation of the 5hmC metabolism associated enzymes such as Tet1/2/3 or Uhrf1/2 was not performed to further investigate the sevoflurane-induced effect of learning and memory impairment as well as 5hmC loss in POCD model.

Overall, the current data suggest that sevoflurane may lead to the suppression of Uhrf2 and induce the loss of global 5hmC in the hippocampus and amygdaloid nucleus, thereby impairing the learning and memory ability of mice with POCD. The present study revealed a novel connection between 5hmC, which is an important biomarker of memory and POCD. The current findings may provide a new biomarker to target or inform the development of a new anesthetic to reduce the incidence of POCD.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ performed experiments and analyzed the data. WX designed the project and drafted the paper.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Fudan University, Shanghai (animal protocol number 2017-32-166) and all animal protocols were conducted following the guidelines accordingly.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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