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Hydroxymethylation in learning and memory

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La science, comme l'amour, est aveugle. Voilà pourquoi elle se plaît à procéder par tâtonnements.

[Jean O'Neil]

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Introduction

1 A brief background on memory

The ability to learn from experience, retain information over time and recalling it are the key functions of human cognitive system. The pioneering work of Miller and colleagues contributed in inaugurating the modern era of memory research. They studied the effect on memory of a bilateral medial temporal lobe resection, performed to discard epilepsy in a patient named H.M., that exhibited one of the most profound anterograde amnesias ever discovered (Scoville and Milner, 1957). Descriptions of H.M established memory as a distinct cerebral function, dissociated from other intellectual functions, and led to the understanding of how memory is organized in the brain. The major structures identified as being involved in memory mainly comprise the hippocampus and much of the parahippocampal gyrus (Scoville and Milner, 1957).

Not only did H.M. largely contribute into the discovery of the anatomy of memory, but also continuous study of H.M. supported the fundamental distinction between different types of memory advocated by James, mainly immediate memory and long-term memory (James, 1890; Wickelgren, 1968). Plus, the experimental demonstration of preserved learning in H.M. patient (Corkin, 1968; Milner, 1962), allowed further characterization of the memory systems in the brain (declarative and procedural memory). Declarative memory is defined as memory in the common sense i.e. knowledge for facts and events, which is known to require the medial temporal lobe (hippocampus and the adjacent entorhinal, perirhinal, and parahippocampal cortices) (Cohen and Squire,

1980; Schacter and Tulving, 1994). Procedural memory involves perceptual and motor skills i.e. skill-based knowledge. While declarative memory engaged the hippocampus, non-declarative memory required other brain regions such as the cerebellum, the amygdala etc. Memory is a complex process that has several temporal phases, which include short-term memory (STM), lasting minutes to hours, and long-term memory (LTM), lasting hours to days or even years.

Neurobiologists generally agree that "memory is a thing in a place in a brain" but the biological processes underlying the way the brain encodes, stores, retrieves, and modifies memory are not fully understood (Martinez and Derrick, 1996). Kandel and colleagues provided strong evidence that learning results from changes in the strength of synaptic connections between interconnected neurons, modulated by the release of transmitters (Castellucci and Kandel, 1974). The application of multiple pulses of serotonin at the sensory-motor neuron synapse was shown to produce a long-term increase in synaptic strength while only one pulse produced a transient synaptic enhancement (Montarolo et al., 1986). This physiological modulation of synaptic efficacy and neural excitability is referred to as synaptic plasticity and supports the cellular connectionist approach proposed by Ramon y Cajal's. The current memory hypothesis states that activity-dependent synaptic plasticity is induced at the appropriate synapse during memory formation (Martin et al., 2000).

But what are the molecular mechanisms underlying STM and how is STM converted to LTM? Molecular studies on STM and LTM have revealed that these two temporal phases of memory have different underlying mechanisms. One of the main differences is that STM depends on existing proteins and requires their covalent modification, while LTM needs the synthesis of new proteins (Kandel, 2001). The processes that induce protein synthesis upon learning have been studied in different animal models, and one of their components to be initially identified is cyclic AMP (cAMP). cAMP is a second messenger present

in most cells, and can activate various enzymes in signaling pathways. In neurons, it is released when a neurotransmitter binds to its cognate receptor upon neuronal activation. Intracellular cAMP then activates different enzymes, in particular protein kinases, such as protein kinase A. The transcription factor cyclic AMP responsive element binding protein (CREB) is one of cAMP ultimate targets. The signaling cascade initiated by cAMP leads to the phosphorylation of CREB by protein kinases, particularly phosphorylation at serine 133 which is necessary to activate transcription (Gonzalez and Montminy, 1989). An activated CREB can mediate the expression of several genes involved in strengthening of existing synapses and the growth of new synaptic connections (Glanzman et al., 1989). Several key studies have provided experimental evidence in favor of the involvement of cAMP and CREB in memory. When injected in the cell body of sensory neurons collected from *Aplysia* and known to be involved in the gill-withdrawal reflex, cAMP was found to strengthen synaptic transmission (Brunelli et al., 1976). Further injection of the cyclic AMP responsive element (CRE) sequence into the nucleus of sensory neurons was observed to block long-term facilitation but did not affect short-term facilitation in *Aplysia* (Dash et al., 1990). Evidence for the role of CREB came from genetic models, in particular knock-out mice deficient for CREB. In contextual fear conditioning, a form of associative learning, CREB mutants were found to have LTM impairment when tested 24 hours but not 30 minutes after training whereas STM was intact (Bourtchuladze et al., 1994). Overall, these studies identified CREB as a key regulator of the conversion of STM to LTM.

The switch to long-term synaptic change and the growth of synaptic connections has been shown to be constrained by memory suppressor genes such as CREB2, which means that the nervous system undergoes constant molecular turnover (Abel and Kandel, 1998). Protein phosphatases are another proposed category of memory suppressors. These molecules act together with protein kinases and form

a molecular balance that controls neuronal signaling and synaptic efficacy (Mansuy and Shenolikar, 2006). Synaptic transmission and plasticity is positively regulated by phosphorylation of specific substrates by protein kinases and negatively by dephosphorylation of protein phosphatases (Mansuy et al., 1998). In that context, protein phosphatases such as the protein phosphatase 1 (PP1) was identified as a molecular constraint on learning and memory by limiting memory acquisition and favoring memory decline (Genoux et al., 2002). Indeed, inhibition of nuclear PP1 was shown to improve memory performance and facilitate long-term potentiation in a transcription-dependent fashion (Graff et al., 2010). These findings highlight an important role for PP1 in the regulation of gene transcription in LTM and synaptic plasticity in the adult brain.

To understand the molecular context underlying learning and memory, one must first understand how single molecules or association of molecules are able to retain information over time in the brain despite their rapid turnover within a cell (Price et al., 2010). It turns out it is not a single "memory molecule" but a cascade of events inside a cell that represents memory.

2 Epigenetics in learning and memory

Only recently, experimental evidences pointed toward a relevant role of epigenetic mechanisms in synaptic plasticity and memory formation. As the epigenome is at the interface of the cellular environment and the genome, epigenetic mechanisms could therefore be considered as consistent with a molecular storage device for memory. Plus, these modifications are thought to be stable and are able to regulate gene expression, in particular memory related genes.

2.1 Introduction to epigenetics

A new sub-discipline of genetics known as epigenetics has recently been highlighted through the discovery of molecular mechanisms underlying cell differentiation and development. Numerous definitions have been described in the literature but the most general one refers to a set of self-perpetuating, post-translational modifications of DNA and nuclear proteins that produce lasting alterations in chromatin structure as a direct consequence, and lasting alterations in patterns of gene expression as an indirect consequence (Levenson and Sweatt, 2005). The molecular mechanisms of epigenetics mainly involve histone and DNA tagging. Chromatin is considered as a dynamic structure that can integrate hundreds of signals from the cell surface, followed by the execution of the resulting transcriptional response. In eukaryotes, chromatin is organized into nucleosome core particles that consist of approximately 147 bp of DNA and an octamer of histones (typically, two of each core histones: H2A, H2B, H3 and H4) (Figure 1A). The amino N-termini of histones are subject to post-translational modifications, mainly acetylation, methylation, ubiquitylation and phosphorylation (Figure 1B). Epigenetic modifications can occur at histone tails

or directly on DNA via the DNA methylation process, which leads alterations of chromatin structure. In most cases, it ultimately results in significant down regulation of transcription as it hampers the binding of transcription factors to regulatory elements. These modifications are mediated by DNA methyltransferases (DNMTs) and consist in the attachment of a methyl group to the 5' position on the cytosine base (Chen et al., 1991). For methylation to occur, cytosine residues must usually be followed by a guanine residue (Cedar et al., 1979).

2.2 DNA methylation and memory formation

DNA methylation is considered as the prime culprit epigenetic modification because of its long-lasting and self-perpetuating capacity and its major role in transcription regulation (Day and Sweatt, 2010), although posttranslational histone modifications, mainly histone acetylation and phosphorylation are also thought to be involved in learning and memory processes (Gräff and Mansuy, 2008; Saab and Mansuy, 2011). Previous works studied the ability of behavioral learning to induce modifications in DNA methylation profile and its involvement in memory processes. For example, a rapid modification of DNA methylation pattern was noted within the promoters of both *reelin* and *brain-derived neurotrophic factor* (*bdnf*), genes implicated in the induction of synaptic plasticity in the adult hippocampus, in response to DNMT activity inhibition (Levenson, 2006).

Furthermore, the expression of DNMTs has been reported to be up-regulated in the adult rat hippocampus following contextual fear conditioning, task used as learning paradigm, while inhibition of DNMTs blocks memory formation (Miller and Sweatt, 2007). This increase in DNMT level after learning is also associated

with an increase in the methylation of *pp1*, a negative constraint on memory formation, and a reduction in the methylation level of *reelin*, a gene known to positively regulate synaptic plasticity and memory. Theses changes in methylation are further associated with a decrease in *pp1* gene expression level and conversely, an increase in *reelin* gene expression level (Miller and Sweatt, 2007). Additionally, *bdnf* promoter also undergoes changes in DNA methylation after fear conditioning, particularly the exon IV that showed decreased methylation associated with an increase in mRNA that returns to baseline 24 hours later (Lubin et al., 2008). Altogether, these findings suggest that DNA methylation is dynamically regulated in the brain, in response to learning, and that it could play a crucial role in regulating the expression of synaptic plasticity and memory related genes.

However, these dynamic DNA methylation changes in response to learning do not corroborate the initial hypothesis that stated methylation as a permanent marker for a LTM device. Indeed, the methylation levels of *pp1* and *reelin* both returned to baseline within 24 hours of training, indicating how dynamic theses modifications are (Miller and Sweatt, 2007). Although, the hippocampus is essential for the consolidation of long-term memories, it is widely believed that LTM traces are stored in the cortex (Weinberger, 2004). Therefore, recent studies focused their attention on the cortex, and could provide strong evidence that DNA methylation persists over time in cortical neurons after training (Miller et al., 2010). This observation is also consistent with the hippocampal/neocortical interactions theory of memory formation, emphasizing the importance of interactions between the hippocampal and cortical regions in which memory traces are stored (Wang and Morris, 2010). Thus, DNA methylation may occur as a dynamic mechanism during memory formation but also remain stable thereafter to maintain those memories. Overall, these findings provide strong insights for the involvement of DNA methylation in maintaining memories.

3 The discovery of a new DNA mark: the sixth base

In the past decade, the epigenetic community has drawn outstanding attention to the fifth base, 5-methylcytosine (5mC). However, a sixth base, 5-hydroxymethylcytosine (5hmC) has been recently discovered, which results from the oxidation of the 5-methyl group of a methylated cytosine. Although, the presence of 5hmC in mammalian DNA has already been described forty years ago in brain and liver tissues (Penn et al., 1972), only recently has the work of Kraucionis and Heintz brought it back to life with the identification of 5hmC in Purkinje neurons using mass spectrometry (Kriaucionis and Heintz, 2009). Following this unexpected finding, several studies focused on the detection of 5hmC in different tissues and cell types. It appears to be present in many different tissues, in particular in the central nervous system (Globisch et al., 2010; Münzel et al., 2010; Song et al., 2010). Globisch and colleagues precisely determined the amount of 5hmC in different mouse tissues. Surprisingly, they found physiologically relevant amounts of 5hmC in all investigated tissues, with the highest levels in neuronal tissues (mainly cerebral cortex, brainstem and cerebellum), suggesting 5hmC presence in a tissue specific manner (Globisch et al., 2010). This finding confirmed the initial observation of Kraucionis and colleagues, where they indicated 5hmC to be enriched in the brain with higher abundance in the cortex and the brainstem (Kriaucionis and Heintz, 2009).

Furthermore, 5hmC could be detected in nuclear DNA of mouse embryonic stem cells (ESCs) and Tahiliani et *al.* using computational search discovered TET proteins, a family of enzymes that most likely are capable of specifically catalyze the conversion of 5mC to 5hmC (Tahiliani et al., 2009). This hypothesis was further confirmed *in vitro* and in cultured cells. The discovery of TET proteins suggests a possible dynamic regulation of 5mC into 5hmC, in a similar way to DNMTs activity (Figure 2). Whereas 5mC is one of the best-characterized DNA

modifications, little is known about the function of 5hmC itself. The precise functions of DNA hydroxymethylation are not yet understood, however the recent discovery that 5mC can be further oxidized to 5hmC due to TET activity, has led to the idea that 5hmC is related to epigenetic reprogramming. Indeed, 5hmC was proposed to regulate the binding of proteins to DNA and thus, the recruitment of histone modifying enzymes, leading to chromatin changes associated with gene repression or activation. The demonstration came from Valinluck and colleagues, showing that 5hmC is indeed able to prevent methyl CpG binding protein 2 (MeCP2) from binding to DNA, once 5mC has been converted into 5hmC (Valinluck et al., 2004), and to inhibit the activity of the human methyltransferase DNMT1 from methylation of target cytosine (Valinluck and Sowers, 2007). Consequently, the inhibition of DNMT activity may interfere with methylation maintenance during cell division, leading to passive demethylation. Related to this, 5hmC was shown to be present ESCs and influence self-renewal and maintenance (Ito et al., 2010). It is also possible that 5hmC gets specifically recognized by yet-unknown proteins and induces changes in gene transcriptional level.

Moreover, the link between 5hmC and epigenetic events may be mediated through the possible active demethylation process, even if it is the subject of controversial discussions. Indeed, Guo and colleagues elegantly demonstrated that TET1 promotes DNA demethylation in the adult brain. The overexpression of TET1 in human cells was shown to trigger the reactivation of a methylation-silenced plasmid reporter and to promote region-specific DNA demethylation of multiple endogenous genomic loci (Guo et al., 2011). This phenomenon was shown to be a direct result of 5-hydroxymethylation of cytosine.

A further investigation into the mechanism underlying 5hmC-mediated demethylation led to a model wherein 5mC is first oxidized to 5hmC by TET proteins and then deaminated by AID/APOBEC deaminases into 5-hydroxyuracil (5hmU). The latter can be excised by 5hmU glycosylases and finally repaired by the components of the base excision repair (BER) pathway (Figure 3). A chemically attractive alternative pathway has been postulated, where the hydroxymethyl group is further oxidized into a formyl or carboxyl group followed by either deformylation or decarboxylation. Indeed, TET proteins can generate 5-formylcytosine and 5-carboxylcytosine (5caC) from 5mC in an enzymatic activity–dependent manner (Figure 3). These two previously unknown cytosine derivatives were found to be present in genomic DNA of mouse ES cells and mouse organs (Ito et al., 2011). Another study demonstrates that 5hmC in DNA are oxidized to 5caC by TET enzymes *in vitro* and in cultured cells. Then, 5caC is specifically recognized and excised by thymine-DNA glycosylase (He et al., 2011). Altogether, these findings provide some insights about a potential role of 5hmC in the active DNA demethylation process.

Furthermore, the presence at physiologically relevant levels and in a tissue-specific manner in the brain has tempted researchers to hypothesize that this DNA modification could have a significant function in the adult brain. Song et *al*. quantified the 5hmC content at different stages of the mouse cerebellum development and observed a gradual increase in the level of 5hmC from postnatal day 7 to adult stage, suggesting a potential role of 5hmC in brain development (Song et al., 2010). The initial discovery from Kraucionis and colleagues of 5hmC enrichment within the brain is supportive of a possible role in regulation of neuronal functions (Kriaucionis and Heintz, 2009).

The major difficulty is to overcome the actual technological barriers. Indeed, due to the close similarity of 5hmC and 5mC, the commonly used bisulfite conversion protocol that provides a methylation profile at single-base-pair resolution, has proven incapable of distinguishing between these two DNA modifications (Jin et al., 2010). A second widely used method is the combination of specific endonucleases with different sensitivities to methylated cytosines. At present, no restriction enzymes have been identified as being able to discriminate between 5mC and 5hmC (Nestor et al., 2010). However, an indirect enzymatic approach has been developed using the T4 phage glucosyltransferase to add a glucose moiety to 5hmC, followed by an enzymatic digestion including two isoschizomers with differential methylation sensitivity (Davis and Vaisvila, 2011). As an alternative, the conventional methylated DNA immunoprecipitation (MeDIP) protocol (Mohn et al., 2009) has been adopted to immunoprecipitate DNA enriched for 5hmC using a specific 5hmC antibody.

Using this technique, a recent study mapped 5hmC genomic distribution from human brain frontal lobe tissue. The major outcome of this study emphasized 5hmC as being more abundant in genes than 5mC, in particular within promoters, whereas it was largely absent from intergenic regions (Jin et al., 2011). In the mouse cerebellum, 5hmC was found to be essentially present in gene bodies and gene proximal regions (Song et al., 2010). Genome-wide mapping of 5hmC in mouse ESCs led to the observation that 5hmC is enriched within exons and near the transcription starting site (TSS) (Ficz et al., 2011; Pastor et al., 2011; Williams et al., 2011). Ficz and colleagues investigated the profile of 5hmC and 5mC in mouse wild-type and mutant ESCs, and determined that whereas 5mC in under-represented at promoters, 5hmC is enriched and also associated with increased transcriptional level (Ficz et al., 2011). Altogether, these findings strongly suggest a potential role of 5hmC in epigenetic regulation of gene expression.

4 Research project: involvement of hydroxymethylation in learning and memory

Given the relative abundance of 5hmC in the brain, including areas crucial for learning and memory formation, and the emerging role of epigenetic regulation in these same processes, this study explores the potential role of 5hmC in the establishment and storage of fear memory in adult mice. The specific questions we ask include: is there a specific regulation of 5hmC after learning and during memory formation? How do alterations in 5hmC profile, if any, relate to corresponding changes in 5mC levels after learning and during memory formation? Do these changes in 5hmC and 5mC profile correlate with gene expression? To answer these questions, we focus on two candidate genes, namely *pp1* and *creb1,* two genes that act respectively, as positive and negative regulators of memory. Combined analyses of the profile of cytosine hydroxymethylation and methylation at the promoter of *creb1* and *pp1* were performed in the hippocampus after fear conditioning, task used as a learning paradigm. For this purpose, an immunoprecipitation protocol followed by quantitative polymerase chain reaction (qPCR) analysis was applied, with which 5hmC and 5mC profiles of the selected genes are analyzed.

Materials and Methods

Animals. Wild-type adult mice (3-8 months old) were used for the experiments. Mice were maintained in standard conditions under a reversed light cycle (dark phase, 7 a.m. to 7 p.m.), and in accordance with the Federation of Swiss Cantonal Veterinary Office and European Community Council Directive (86/609/EEC) guidelines.

Contextual fear conditioning. The animals were handled for three days before the start of behavioral experiments and accustomed to the test environment by a brief (lasting 5 minutes) daily exposure to novel chambers (Figure 4). For contextual fear conditioning, animals were placed into the fear conditioning chamber (context) with a metal-grid floor and allowed to explore the chamber for 2 minutes, after which they received three electric foot-shocks (magnitude=0.3A; duration=1s; interstimulus interval=60s). The animals were removed from the chamber 1 minute after the last foot-shock. Controls animals were exposed to the same fear conditioning chamber for the same duration as the fear-conditioned animals but received no foot-shock and were sacrificed 30min later. Fear-conditioned animals were sacrificed at different time point i.e. 30 minutes after training, 3 hours after training, 24 hours after training and 30 minutes after testing long-term memory. During the long-term memory test conducted 24 hours after training, the mice were exposed to the fear conditioning chamber for 5 minutes, in the absence of foot-shock. The freezing response, defined as the absence of movement (crouching position for more than 1s), except for breathing, was measured as a measure of fear memory and reported as a percentage of time. All behavioral experiments were video-recorded and, in addition to manual

scoring of freezing response, an automatic scoring of freezing response tracked the animals (tracking system from TSE Systems, Frankfurt Germany).

Brain dissection. The animals were killed by cervical dislocation at different time points and the brains were extracted. Different brain regions (olifactory bulb, prefrontal cortex, cortex, basal ganglia, hippocampus and cerebellum) were dissected on a cold glass surface. The tissues were snap frozen in liquid nitrogen and subsequently stored in -80˚C until further processing.

HydroxyMethylated/Methylated DNA Immuno-Precipitation. hMeDIP and MeDIP (Figure 5 in the appendix) were adapted from the protocol described by Mohn and colleagues (Mohn et al., 2009). Mice are distributed in different groups i.e. group 1: control mice (n=3), group 2: fear conditioned mice sacrificed 30 minutes after training (n=4), group 3: fear conditioned mice sacrificed 3 hours after training (n=4), group 4: fear conditioned mice sacrificed 24 hours after training (n=4) and group 5: fear conditioned mice tested after 24 hours and sacrificed 30 min after testing (n=4). Hippocampus from mice owing to the same group was mixed producing in total five pooled samples for DNA extraction and immunoprecipitation.

***Extraction of genomic DNA*.** DNA samples were homogenized in lysis buffer (20 mM Tris pH 8, 4mM ETDA, 20 mM NaCl, 1% SDS) by drawing the homogenate ten times through a 22-G needle attached to a 1 ml syringe. After centrifugation, the supernatant is incubated three to five hours at 55˚C with proteinase K. DNA is extracted using phenol:chloroform: isoamyl alcohol 25:24:1 v/v (Sigma) and precipitated with 3M sodium acetate pH 5.2 and two volumes ice-cold 100% ethanol.

Sonication of genomic DNA. 20 µg of DNA were diluted 400 µl in TE buffer (Tris 10 mM, EDTA 1mM pH 8.0) and sonicated on ice using a Sonopuls Bandelin (10 cycles of 10s each, 20% amplitude). These parameters were optimized to generate DNA fragments between 300 to 1000 bp, which was confirmed by running the DNA on a 2 % gel agarose. The sonicated DNA was not further precipitated.

Immunoprecipitation (IP) of hydroxymethylated and methylated DNA. 30 pg of control hydroxymethylated, methylated and unmethylated dsDNA sequences (Diagenode, Liège, Belgium) were mixed with 2 µg of genomic DNA before immunoprecipitation. 1 µg of the sonicated DNA was left untreated to serve as input control. Immunoprecipitation was performed by incubating each samples with 10 µg of mouse monoclonal antibody against 5-methylcytidine (Eurogentec, Seraing, Belgium), or with 10 µg of mouse monoclonal anti-5hmc antibody (Active Motif, Carlsbad, USA), or an equivalent amount of normal rabbit IgG (Millipore, Strasbourg, France) as negative control overnight at 4°C. On the next day, DNA-antibody complexes were incubated with 40 µl Dynabeads M-280 Sheep anti-mouse IgG (Invitrogen, Lucerne, Switzerland) for 2h at 4°C and sequentially collected with a magnetic rack followed by three washings with IP buffer (10 mM Na-Phosphate pH 7.0, 140 mM NaCl, 0.05% Triton X-100). After proteinase K digestion (70 µg; 3 h at 50°C), DNA was extracted by phenol/chloroform/isoamyl alcohol, ethanol-precipitated and resuspended in 60 µl TE.

Targeted regions. The promoter of the human *cyclic AMP-response element binding 1* (*creb1*) gene was found to be hydroxymethylated on chromosome 2 between 208100735-208101090 bp (Jin et al., 2011). Using a Blast z sequence alignment program, the homologous region in mouse was found to be located on

chromosome 1 between 64251477-64251744 bp (see Figures 3 and 4 in the appendix). For the following experiment, this region within *creb1* promoter was targeted. Knowing that 5hmC is preferentially present at promoters with high or intermediate CpG content (Pastor et al., 2011), an online program named Methprimer was used to predict the occurrence of CpG islands within *pp1* and *gapdh* promoters. The presence of a CpG island was revealed around the ATG of *pp1γ* and *gapdh*. Primers were designed within and around these two target regions (Figure 5).

Quantitative PCR (qPCR). Immunoprecipitated DNA was subjected to quantitative real-time PCR using specific primers for approximately 200 bp segments corresponding to *creb1* and *pp1* promoters. Each reaction was performed using the commercial SYBR green master mix (FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and MgCl2, Roche) on a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Equal amounts of DNA were quantified in triplicate for each sample. The cycling conditions used were the following: 5 min at 95˚C, 45 cycles of 30 s at 95˚C, 40 s at 60˚C, and 1 min at 72˚C and data were collected using an absolute quantification. The efficiency of hydroxymethylated and methylated IP of particular genomic locus (i.e. *creb1* and *pp1* promoters) can be calculated from qPCR data and reported as a percentage of starting material (Input) using the following formula: % Input = $2^{(Ct\ Input - Ct\ MeDIP)}$ x dilution factor x 100. Primer sequences are presented in the appendix (Figure 1).

Results

1 Validation of 5hmC detection method (hMeDIP)

DNA extraction and sonication. Cortical DNA was extracted from naive wild-type mice using a simple phenol-chloroform based protocol. As DNA fragments ranging from 300 bp to 1000 bp are required, different sonicator settings were applied i.e. 5 times 20s at 25% amplitude (1); 10 times 20s at 25% amplitude (2); 5 times 10s at 20% (3); 10 times 10s at 20% amplitude (4). The sheared DNA size was systematically checked on agarose gel to ensure equal sonication between samples. Settings 1 and 2 produce DNA fragments between 100-800 bp, while setting 3 generates fragments of about 500 to 1500 bp (Figure 6). However, setting 4 generates DNA fragments within the expected size range (300-1000bp). Thus, DNA sonication was performed using setting 4 i.e. 10 times 10s at 20% amplitude.

Detection of hydroxymethylated control sequences in mouse cortical DNA. The IP has been optimized to specifically select and precipitate hydroxymethylated fragments. Standard commercial hydroxymethylated, methylated and unmethylated DNA fragments were equally mixed within the starting DNA material. The use of exogenous DNA sequences allows the characterization of the immunoprecipitation efficiency and specificity. The IP was performed with the recommended starting DNA amount of 4 µg (sample 1) and 2 µg (sample 2). Using primer pairs that specifically amplify the exogenous DNA sequences, a quantitative PCR is performed as reported in the materiel and methods section. Normalization against the input (non precipitated DNA) is performed so that a

percentage of recovery is calculated for each DNA control. The anti-5hmc antibody seems specific, as it is able to specifically recognize the hydroxymethylated DNA fragment, while it does neither respond to the presence of methylated, nor to unmethylated fragments (Figure 7). The percentage of recovery reached up to 13%, which is acceptable regarding the recovery of about 14% proposed by the company providing the antibody (Active Motif, Carlsbad, USA). Furthermore, both immunoprecipitations seem reproducible as a similar recovery percentage was yielded regardless of the initial DNA amount.

Analysis of hydroxymethylation profile within *pp1* and *creb1* promoters in mouse cortical DNA. As the immunoprecipitation demonstrated specificity and efficiency, further analyses were performed using primers that target endogenous genes of interest i.e. *creb1* and *pp1*. (Figure 8A). A similar pattern of hydroxymethylation could be observed in both cortical samples, which attests of the IP reproducibility between samples (Figure 8B). Interestingly, some regions seem to be more hydroxymethylated compared to others within the same gene. Indeed, regions 2 and 3 of *pp1* reproducibly reached the highest value of percentage recovery in both samples. Furthermore, the IgG antibody is completely unspecific as the percentage of recovery is about 0.01%. Thus, this protocol enables to specifically and reproducibly immunoprecipitate hydroxymethylated cytosines.

2 Contextual fear conditioning

Animals were trained using a rodent contextual fear conditioning paradigm where a novel context (fear conditioning chamber) is associated with a series of grid foot-shock. Contextual fear conditioning was chosen because it is hippocampus-dependent and requires *de novo* gene transcription. To investigate the potential changes in hydroxymethylation and methylation profiles after learning, a time-course protocol was used i.e. mice were sacrificed at different time-points after training (i.e. 30 min, 3h, 24h). Control animals were placed in the same training chamber but did not receive any shock. Figure 9A represents the protocol used for fear conditioning. The first two minutes, mice were allowed to explore the chamber, after which they received three electric foot-shocks. The animals were removed from the chamber 1 minute after the last foot-shock. The freezing response in fear conditioned mice before shock (0 to 2 min) and immediately after foot-shocks (4 to 5 min) is compared to non-conditioned mice (average of five independent group of mice). The freezing response of control mice stays stable over time, while the freezing of fear conditioned animals increases in response to the electric stimulus, showing a direct behavior effect (Figure 9B). One group of mice was re-exposed to the training chamber 24 hours after training without receiving any foot-shocks. These animals retrieved memory for the context as showing by the high percentage of freezing response (≈55%), attesting that the contextual fear conditioning protocol is functional in inducing learning and fear memory (Figure 9C).

3 Analysis of 5hmC and 5mC profiles at the promoter of *pp1* and *creb1*

Once 5hmC detection method has been validated, hippocampus samples from fear conditioned and control mice were processed in the following analysis. As methylation is implicated in learning and memory processes (see part 1.b introduction), both methylation and hydroxymethylation profiles at *creb1* and *pp1* promoters were investigated. The combination of hMeDIP and MeDIP allows the performance of both DNA hydroxymethylation and methylation analyses in a DNA sequence-specific context. This dual analysis was developed and optimized together with the use of internal immunoprecipitation controls.

DNA extraction and sonication. As the fear conditioning task is hippocampus-dependent, genomic DNA was extracted from pooled mouse hippocampi. DNA fragments within the expected size range were obtained, using the following sonicator parameters : 10 times 10s at 20% (data not shown).

Detection of DNA control sequences in mouse hippocampal DNA. According to Figure 10, various immunoprecipitation efficiencies were reached among all five immunoprecipitated samples, meaning there is a non-negligeable experimental variability. However, the anti-5hmC antibody seems specific to 5hmC, as it specifically recognizes the hydroxymethylated DNA fragment, whereas it does neither binds to methylated, nor to unmethylated fragments. On the contrary, one can notice the non-specificity of the anti-5mC antibody as it responds to the presence of both methylated and hydroxymethylated sequences, with an even higher percentage of recovery for 5hmC than the anti-5hmC antibody itself. Interestingly, it implies that former methylation profile analyses were incapable of distinguishing 5mC over 5hmC. For the purpose of the current study, it is not that important as we are more interested in the 5hmC profile.

Analysis of DNA hydroxymethylation and methylation profiles within *pp1* and *creb1* promoters in the hippocampus. As previously mentioned, the use of equal amount of exogenous DNA controls in each IP i.e. hydroxymethylated, methylated and unmethylated fragments, allows to characterize the efficiency and specificity of each 5hmC and 5mC immunoprecipitations. Due to the variability of immunoprecipitation efficiency among samples, a normalization method has been implemented in order to enable a proper comparison of both hydroxymethylation and methylation profile changes in response to fear conditioning, excluding the experimental variability. For each single IP, the 5hmC recovery percentage of the gene of interest was normalized to the recovery percentage of the hydroxymethylated control. The same procedure was followed for methylation i.e. the 5mC recovery percentage of the gene of interest was normalized to the recovery percentage of the methylated control, allowing the removal of the experimental variability due to a difference of immunoprecipitation efficiency.

Normalized to DNA controls. Two different regions within *creb1* promoter were investigated, respectively region 2 and region 3 (Figure 11A). According to Figure 11B, the hydroxymethylation level within region 2 of *creb1* promoter considerably grows after 30 min of fear conditioning. Two hours and a half later, a dramatic reduction is observed and the level of hydroxymethylation stays low until testing. Methylation profile shows slight variations over time, which is not as consequent as hydroxymethylation changes in response to fear conditioning. Figure 11C also shows an important increase in 5hmC within region 3 of *creb1* promoter 30 min after fear conditioning, compared to the baseline control level. Subsequently, the level of hydroxymethylation decreases over time until approximately reaching the level of control mice. Interestingly, when the animals are placed back in the chamber and tested for memory retrieval, the level of hydroxymethylation reaches

the highest value again. Concerning methylation patterns within *creb1* promoter, no considerable changes are noticed 30 minutes, 3 hours and 24 hours after fear conditioning compared to control animals. These results suggest a dynamic and direct up-regulation of 5hmC in response to fear conditioning within *creb1* promoter. However, 5mC seems not to be considerably affected.

Here also, two different regions within *pp1* promoter were investigated, respectively region 3 and region 4 (Figure 12A). Hydroxymethylation within region 3 of *pp1* promoter does not exhibit any considerable changes in response to fear conditioning, while methylation exhibits a slight increase after 30 min of fear conditioning following by an overall tendency to reduce (Figure 12B). According to Figure 12C, the hydroxymethylation pattern within region 4 of *pp1* promoter has an overall tendency to slightly decrease in response to fear conditioning compared to the baseline control level. On the contrary, fear conditioning produces an eleven-fold increase in methylation, which subsequently decreases over time until reaching approximately the baseline level. Such a change in methylation within *pp1* promoter has been already discovered in rats (Miller and Sweatt, 2007), validating our detection method. These results suggest a dynamic and direct up-regulation of 5mC in response to fear conditioning within *pp1* promoter. However, 5hmC seems not to be considerably affected.

Normalized to gapdh. In order to access the validity of these results, a second normalization method has been applied. The promoter region of *gapdh*, a housekeeping gene, was used as an endogenous control gene. Two different regions of *gapdh* promoter were investigated i.e. *gapdh* region 1 outside a CpG island (GC content of 51%) and *gapdh* region 2 within a CpG island (GC content of 64%) (Figure 13A). The recovery percentage for 5hmC within *gapdh* promoter was normalized to the recovery percentage for 5hmC within *creb1* or *pp1* promoters for each single IPs. Same procedure was followed for methylation i.e. the recovery percentage for 5mC within *gapdh* promoter was normalized to the recovery percentage for 5mC within *creb1* or *pp1* promoters. Only region 3 of *creb1* and region 4 of *pp1* were investigated and normalized to both regions of *gapdh*. Figure 13B indicates that fear conditioning produces a three-fold increase in hydroxymethylation level within *creb1* promoter, which reaches its culminant point 3 hours after shock. Then, a reduction in hydroxymethylation occurs 24 hours after fear conditioning. When the animal is placed back in the fear conditioning chamber and tested for long-term memory, the hydroxymethylation pattern exhibits a consequent growth. These results are in line with what was observed when normalized against DNA controls. Methylation profile differs from hydroxymethylation as no consequent changes were induced 30min and 3h after fear conditioning. However, 24h after fear conditioning, a strong increase is observed, that stays when the animal is tested back in the same chamber. This effect may be due to a variation in the methylation pattern within both regions of *gapdh* gene that, surprisingly, occurs only 24 hours after fear conditioning and after the testing event. Similar patterns are noticed when region 2 of *gapdh* promoter is used as a control (Figure 13C). Altogether, these results suggest a dynamic and direct up-regulation of 5hmC in response to fear conditioning within *creb1* promoter.

Figures 14B and 14C (top panels) report no considerable variations in *pp1* hydroxymethylation profile, only a slight overall increase when normalized to region 1 of *gapdh*. On the contrary, a nine-fold increase in methylation is induced 30 min after fear conditioning compared to baseline, followed by a subsequent tendency to reduce (Figure 14B, bottom panel). A global similar pattern is observed when region 2 of *gapdh* is used as a control (Figure 14C, bottom panel). Thus, the methylation level particularly grows 24 hours after fear conditioning and after testing, no matter which region of *gapdh* is used for normalization. These results suggest a dynamic and direct up-regulation of 5mC in response to fear conditioning within *pp1* promoter.

These analyses provided preliminary evidence that 5hmC is up-regulated at *creb1* promoter in response to fear conditioning and fear memory consolidation, while in contrast 5mC was increased at *pp1* promoter. These initial results suggest a dynamic regulation of 5hmC in the hippocampus in response to learning and fear memory.

Discussion

The learning and memory field has recently recognized the importance of epigenetics underlying memory formation. While former research has focused on the role of transcription factors in regulating synaptic plasticity and memory, a growing area is highlighting the potential implications of epigenetics mechanisms in the transcriptional regulation underlying memory.

The recent discovery of 5hmC, a modified DNA base that derived from oxidation of 5mC by TET1 enzymes, contributed to the awareness of previously undiscovered DNA modifications with potential physiological significance. It has been demonstrated that 5hmC is enriched in the brain and is abundant in areas crucial for learning and memory formation. Given the recently discovered role of 5mC in epigenetic regulation of learning and memory processes, the current study explored the potential role of a new epigenetic mechanism, cytosine 5-hydroxymethylation, in the establishment and storage of fear memory in adult mice. To this end, an immunoprecipitation-based approach was adopted and optimized together with the use of exogenous DNA controls and an endogenous housekeeping gene, *gapdh*. A combined analysis of the profile of cytosine hydroxymethylation and methylation at the promoter of *creb1* and *pp1,* two genes that act respectively as positive and negative regulators of memory, was performed in response to fear conditioning, task used as a learning paradigm.

We observed using direct molecular methods that DNA hydroxymethylation level seems to be rapidly and dynamically regulated in the hippocampus following the learning paradigm of contextual fear conditioning. These analyses provided

preliminary evidence that 5hmC is up regulated at *creb1* promoter in response to fear conditioning and fear memory consolidation. This effect is observed 30 min after fear conditioning, suggesting a dynamic and fast effect after which the level of 5hmC undergoes a temporal diminution. Given the postulate that 5hmC is involved as an intermediate in the process of active DNA demethylation (Guo et al., 2011), the observed up-regulation of 5hmC in response to fear conditioning would suggest that *creb1* promoter undergoes demethylation in response to learning and memory formation. As methylation is associated with repression of gene expression, the demethylation of *creb1* promoter may lead to transcriptional activation, required for memory formation and consolidation. This is in line with the known role of *creb1* as a transcriptional activator of synaptic plasticity and memory-related genes required for memory formation and consolidation (Silva et al., 1998). An up-regulation of 5hmC within *creb1* promoter occurs in response to memory retrieval as well, further strengthening the hypothesis according to which *creb1* undergoes demethylation consequently followed by transcriptional enhancement. Indeed, this transcription factor is not only required for the initial consolidation of long-term memory, but also for consolidation of reactivated conditioned fear memory. Although the transcriptional mechanisms necessary for initial memory consolidation and stability after retrieval differ, CREB is required for both (Kida et al., 2002). These findings give some evidence about the possibility of 5hmC to be involved in epigenetic mechanisms that may be implicated in transcriptional regulation underlying learning, memory consolidation and reconsolidation.

Meanwhile, methylation within *creb1* promoter is not considerably affected in response to behavioral training. Importantly, the anti-5mC antibody was not able to discriminate between methylated and hydroxymethylated control sequences (Figure 10). Thus, it is possible that a reduction in 5mC level could have been

masked by a substantial increase in 5hmC level within *creb1* promoter, explaining why methylation level within *creb1* remains stable. The non-specificity of anti-5mC antibodies also carries a wider implication on studies based on immunoprecipitation for methylation profile analysis (MeDIP), as this technique seems to detect 5mC together with 5hmC. This is partly corroborated by the work of Jin and colleagues, which suggests that MeDIP keeps its specificity for 5mC, unless both modified bases are located within the same DNA fragment (Jin et al., 2010). This dissimilarity of results between the two studies is likely due to a difference in the detection limit and sensitivity of the MeDIP downstream analysis method (quantitative PCR versus liquid scintillation counting). It may therefore be important to revisit all known biology of DNA methylation and develop methods able to specifically distinguish cytosine, 5mC and 5hmC preferably in combination with high-throughput sequencing.

Despite the non-specificity of the anti-5mC antibody, we were able to observe a clear and strong up-regulation of 5mC within *pp1* promoter, a negative constraint on memory formation, following learning and memory formation. In parallel, the hydroxymethylation profile does not show considerable changes, implying that only 5mC is regulated within *pp1* promoter after fear conditioning. A study performed by Miller and Sweatt (Miller and Sweatt, 2007) has already demonstrated enhanced methylation in the same stretch of DNA within the *pp1* promoter in response to fear conditioning. Our study adds a supplementary level of details as different time-points after fear conditioning were examined. We could therefore follow the profile of methylation within *pp1* over time and also associate it with different phases of memory. An up-regulation within *pp1* promoter occurs 30 min after fear conditioning, after which it undergoes a temporal diminution, in the manner of 5hmC within *creb1* promoter. The methylation level stays high when the memory is retrieved and consolidated 24

hours later. The increase in pp1 methylation is in line with actual models of memory formation, wherein memory-suppressor genes undergo transcriptional silencing.

Altogether, the increase in *creb1* hydroxymethylation following learning and fear memory formation suggests a certain plasticity of DNA hydroxymethylation in the hippocampus during memory formation. Our findings provide insights that DNA hydroxymethylation, like DNA methylation, may be co-opted by the adult CNS and may functions as an epigenetic mechanism that regulates the transcriptional regulation underlying memory formation and consolidation. This study is the first to present a simultaneous combined analysis of 5hmC and 5mC. These initial results suggest that the regulation of 5hmC in the hippocampus during memory formation may provide a mechanism leading to the demethylation and activation of positive regulators of memory and/or the methylation and silencing of negative regulators of memory formation after training. However, only statistical analyses on qPCR triplicates that quantified one immunoprecipitated sample were achieved. Indeed, each IP was performed on pooled hippocampus samples, disabling the statistical analyses of individual animals. Repetition of the immunoprecipitations will have to be completed in order to verify the significance of these results.

The presence of both DNA marks within *pp1* and *creb1* promoters and its relation with gene expression still needs to be unraveled. Intuitively, it seems that some genes must be activated and other silenced for memory formation to occur. It is therefore tempting to postulate that changes in hydroxymethylation and methylation profiles will correlate with gene expression i.e. methylation will induce transcriptional repression of *pp1* and hydroxymethylation will lead to transcriptional activation of *creb1*. If so, this would suggest that a DNA demethylase must be present in order to counteract DNMT's activity. The

existence of such an enzyme is controversial. However, TET1 proteins are major candidates to take on this role as these enzymes demonstrated demethylation toward 5hmC in the adult brain via the base excision repair pathway (Guo et al., 2011). At present, no clear DNA demethylase has been identified, pointing out the inexistence of one single enzyme but rather a succession of different enzymes with complementary catalytic activities.

Further characterization will require the assessment whether mRNAs for TET1 proteins are up regulated in the hippocampus following learning, testifying for the clear 5hmC increase observed within *creb1* promoter. Similarly, it may be interesting to check whether the level of mRNAs for DNMT enzymes has also increased in response to fear conditioning. Plus, pharmacological inhibition of DNMT activity was shown to block memory consolidation (Miller and Sweatt, 2007). It may become relevant to pharmacologically blocks TET1 activity and examines the consequent effect on memory consolidation. This would definitely give strong evidences that 5hmC is an epigenetic mark that regulates and is regulated by memory formation and consolidation. As future directions, the identification of a potential role of TET in dissociable memory processes could be assessed in temporally and reversibly repressing TET function at key time points in a fear conditioning protocol for example.

The need to overcome the actual technological barriers is high. The development of new methods capable of distinguishing cytosine, 5mC and 5hmC will be the driving force of the field. Epigenetic companies have already developed some detection methods that allow the specific distinction of 5hmC at particular DNA sites in a single nucleotide resolution manner, using a 5hmC tagging and a subsequent enzymatic digestion. Using this method, we were indeed able to characterize the presence of a hydroxymethylated site within *creb1* promoter (data presented in the appendix).

The discovery of TET proteins and 5hmC contributed to the awareness that cytosine in the genome exists in many different forms and thus greatly expanded our knowledge of epigenetics plasticity in the genome. Moreover, the role of epigenetics in learning and memory mechanisms in the adult nervous system are very complex but fascinating. Not only did Kraucionis and Heintz unexpectedly reveal an additional DNA mark but opened an entirely new front in epigenetic research as well and is a guarantee of many more years of unremitting research.

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Appendix

Regions	Forward sequences	Reverse sequences	Amplified fragment (bp)	GC content (%)
CREB region 1	CCTCCCAGTTGGTCCTGTAA	TCGTCCACAAGAGGAGATCA	177	42
CREB region 2	TGAACTAGCCATATGAGGGCAACCA	GCATTACCAGGGTAGCCAGGGGT	193	42
CREB region 3	CCAAAAGCAGGAGCTTTCAC	GGCTGTCCTGGAACTCACTC	211	46
PP1 region 1	TCCAAGAGCTACCTGCCACT	GCTGAATGTCATGCTGGAGA	174	50
PP1 region 2	CAGCTCCGGCTAAATACAGG	TCCACTGTGAGTGGCACATT	190	37
PP1 region 3	ACGTTCCAACCTCCTCCTCT	CGCAAGTTGAAGGTGGACTT	195	58
PP1 region 4	GGCCATCTTGTTCTTCTCGT	CACTCGTCCTCCTTCCTCAC	162	73
GAPDH region 1	TGAGCCTCCTCCAATTCAAC	CCAGGAAGACGCTTGAAAAG	245	64
GAPDH region 2	ACTCGCGGCTTTACGGGTGC	TTTCCGACTGTCCCCGGCCT	173	51

***Figure 1**: Description of primer sequences, size of the amplicon and the associated percentage of GC content.*

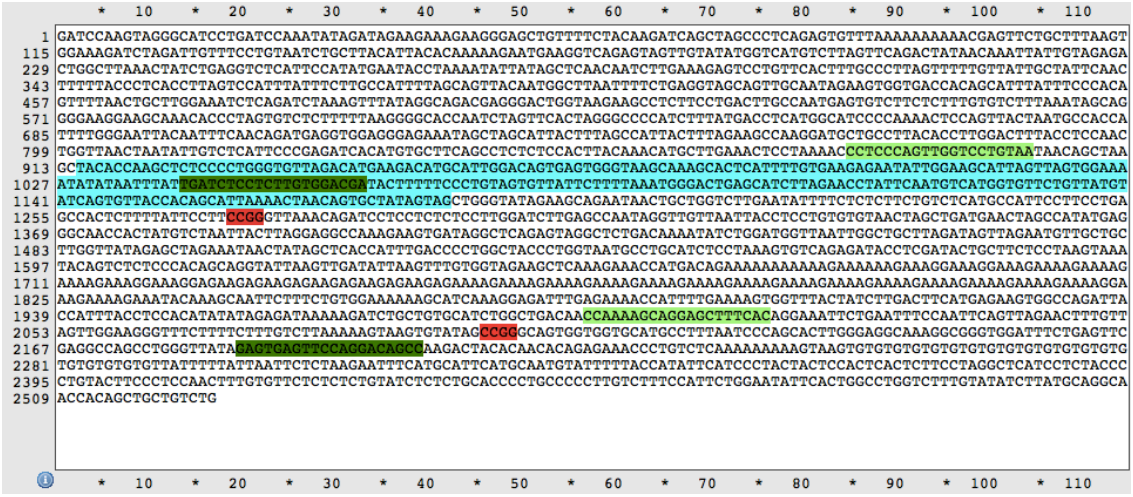

***Figure 2**: Targeted sequence within creb1 promoter. The potential hydroxymethylated sequence is depicted in blue. MspI sites (CCGG) are highlighted in red. Forward primers are highlighted in light-green and reverse primers are highlighted in dark-green.*

Figure 3: Alignment of the potential hydroxymethylated region within creb1 human promoter and the mouse genome using Ensembl Genome Browser (http://www.ensembl.org/index.html). The homologous region in mouse was found on chromosome 1 between 64251477-64251744bp.

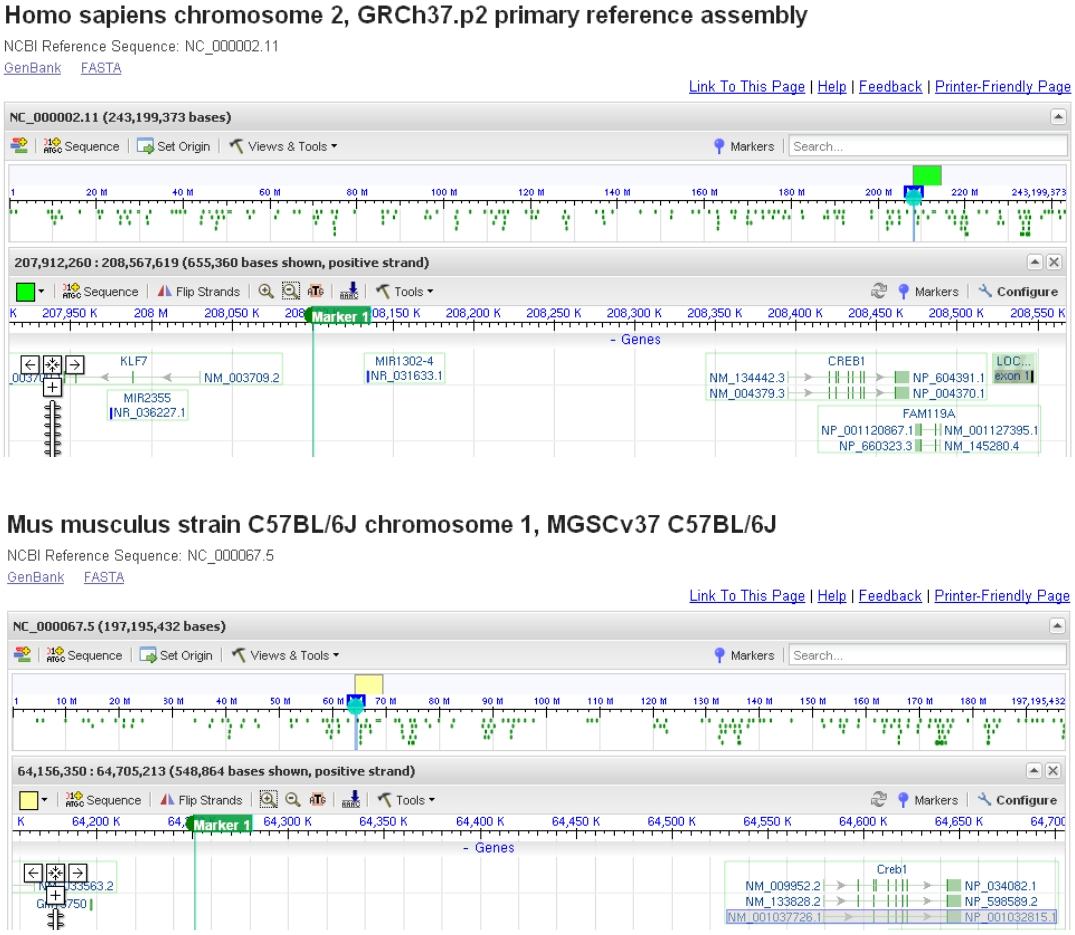

***Figure 4**: Marker 1 (depicted in green) indicates the position of the potential hydroxymethylated region within creb1 promoter in human (A) and in mouse (B).*

MeDIP/hMeDIP workflow

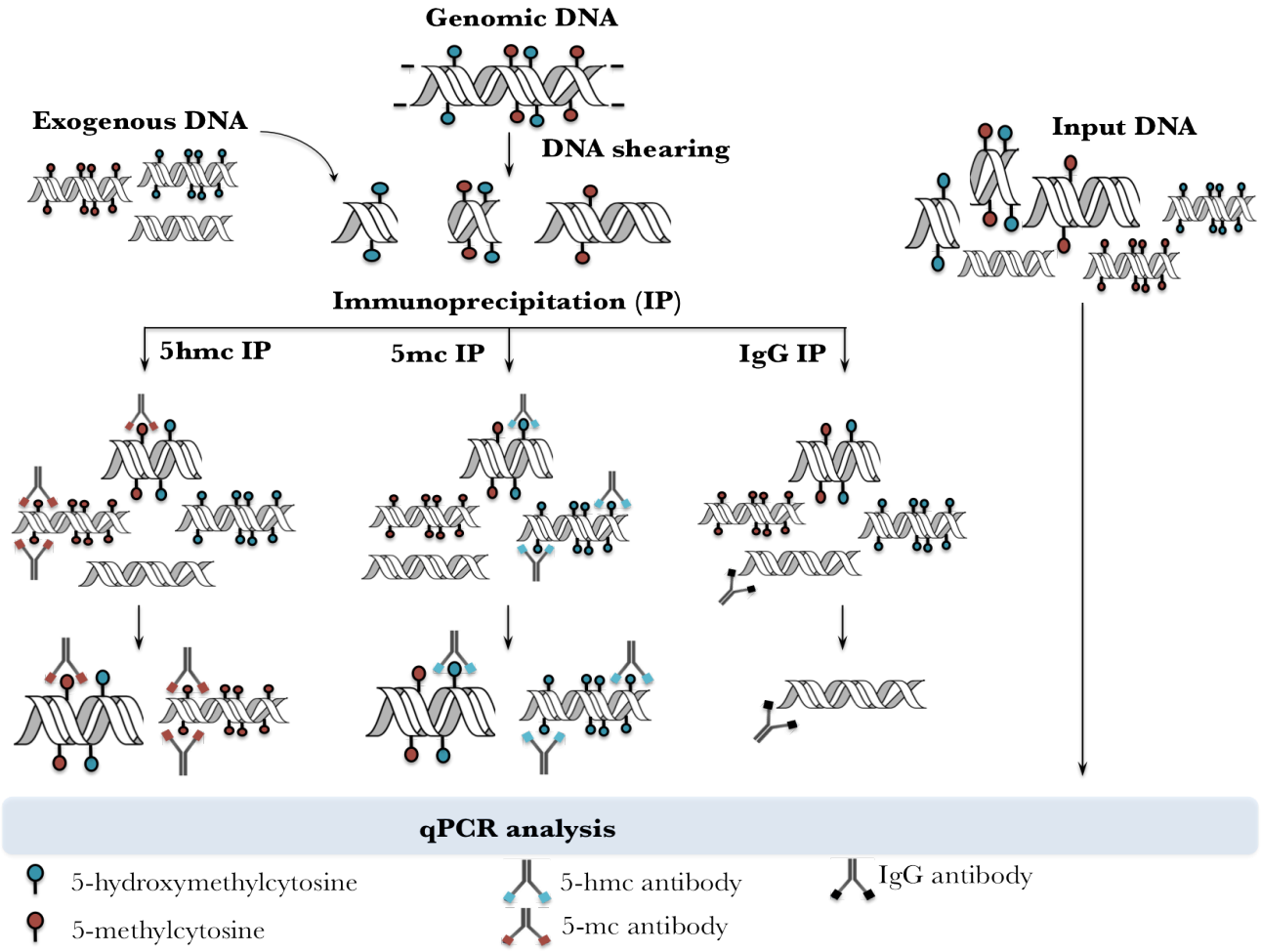

Figure 5: Schematic representation of MeDIP/hMeDIP workflow. *Genomic DNA is sheared in fragments between 300 bp and 1000bp. Then, a specific anti-5mC antibody and anti-5hmC antibody respectively immunoprecipitates fragments containing 5mC and 5hmC. Anti igG antibody is used as negative control. Subsequent analysis is performed by quantifying the immunoprecipitated fragments*

Quest 5-hmC Detection Kit™

Given the recent discovery of 5hmC, few detection methods able to specifically distinguish 5mC over 5hmC are not available yet. However, one commercial enzymatic restriction-based method has been developed and allows a sequence specific detection of 5hmC within DNA using a simple reaction setup. Briefly, a 5hmC glucosyltransferase enzyme specifically tagged 5hmC with a glucose moiety yielding a modified base glucosyl-5-hydroxymethylcytosine (Figure 6). Then, digestion of DNA with a glucosyl-5-hydroxymethylcytosine restriction endonucleases (GSRE) i.e MspI allows the differentiation of 5mC from 5hmC. When 5hmC is glucosylated, the GSRE cannot digest the DNA while it cut DNA when cytosine, 5mC and 5hmC is withinits recognition sequence (Figure 7). Finally, a qPCR is performed to assess locus-specific presence of 5hmC. The presence of 5hmC at the MspI site in the DNA results in a higher amplification efficiency for the glucosylated sample (lower Ct value) relative to the unglucosylated one (higher Ct value).

1 Materiel and methods

Quest 5-hmC Detection Kit™ (Zymoresearch, Irvine, USA). Mouse genomic DNA was processed according to Quest 5-hmc Detection Kit™ protocol. Briefly, 500 ng of DNA, 10X Uridine Diphosphoglucose 1mM and 10X 5-hmC GT reaction buffer were mixed in two separate reaction setups i.e. one reaction comprises 4 units of T4 phage glucosyltransferase (+ glucosylation) and the second without glucosyltransferase (- glucosylation). All reactions were incubated at 37 °C for at least 2 hours for the glucosyltransferase to specifically add a glucose

moiety to each 5hmC residue. Subsequent enzymatic digestion using 30 units of glucosyl-5hmC sensitive endonuclease Msp1 (CCGG) was carried out at 37°C for at least 4 hours in order to specifically cleaved DNA carrying cytosine, 5mC and 5hmC in the recognition sequence whereas glucosyl-5-hmC remains uncleaved. The same procedure is applied on Quest 5-hmC control DNA (double stranded DNA that contains a CCGG site, which carries an hydroxymethylated cytosine). Then, the processed DNA is cleaned with the use of a spin colum DNA clean-up. Subsequent analysis of 5hmC sites is achieved with qPCR (see materiel and methods section).

2 Results

Within creb1 promoter, only two CCGG sites are present (Figure 2 above). One site was found to be hydroxymethylated. Figure 8A represents amplification curves of the glucosylated sample (DNA+G) versus the unglucosylated sample (DNA-G). qPCR amplification of DNA+G sample yields to a higher amplification efficiency compared to DNA-G, suggesting the presence of 5hmC in the targeted MspI site (CCGG). The same difference in amplification efficiency is visible between glucosylated positive control and the unglucosylated postive control (Figure 8B). Melting curve analysis shows the amplification of a single product and no primer dimers, certifying the validity of the results (Figure 8C).

This detection method allows the specific distinction of 5hmC at particular DNA sites in a single nucleotide resolution manner and in our case could indeed distinguish the presence of a hydroxymethylated site within the targeted region of *creb1* promoter.

Abstract

A new DNA mark, 5-hydroxymethylcytosine (5hmC) that derives from oxidation of 5-methylcytosine (5mC) by TET1 enzymes and is thought to provide an intermediate step in DNA demethylation, has recently been discovered. Initial analyses of 5hmC showed that it is enriched in the brain and is abundant in areas crucial for learning and memory formation. Based on these findings, this study explores the potential role of 5hmC in the establishment and storage of fear memory in adult mice. To this end, combined analyses of the profile of cytosine hydroxymethylation and methylation at the promoter of *creb1* and *pp1*, two genes that act respectively, as positive and negative regulators of memory, were performed in the hippocampus after fear conditioning. These analyses provided preliminary evidence that 5hmC is up-regulated at *creb1* promoter in response to fear conditioning and fear memory consolidation, while in contrast 5mC was increased at *pp1* promoter. These initial results suggest a dynamic regulation of 5hmC in the hippocampus during memory formation that may provide a mechanism leading to the demethylation and activation of positive regulators of memory and/or the methylation and silencing of negative regulators of memory formation after training.

Une nouvelle modification de l'ADN, 5-hydroxyméthylcytosine (5hmC), résultant de l'oxydation d'une 5-méthylcytosine (5mC) par les protéines TET1 et considérée comme un intermédiaire dans le processus de déméthylation, a été récemment découverte. Des analyses préliminaires de 5hmC montrent que ce résidu est enrichi dans le cerveau et abondant au niveau de régions essentielles à la formation de la mémoire. De ce fait, cette étude explore les rôles potentiels de 5hmC dans la mise en place et le stockage de la mémoire de la peur chez la souris adulte. Ainsi, une analyse conjointe du profil d'hydroxyméthylation et de méthylation au sein des promoteurs de *creb1* et *pp1,* deux gènes qui agissent respectivement en tant que régulateur positif et négatif de la mémoire, a été réalisée dans l'hippocampe après conditionnement de peur contextuelle. Ces analyses indiquent que 5hmC est sur-régulé au sein du promoteur de *creb1* en réponse au conditionnement à la peur et à la consolidation de la mémoire alors que le niveau de 5mC augmente au sein du promoteur de *pp1*. Ces résultats préliminaires suggèrent une régulation dynamique de 5hmC dans l'hippocampe pendant la formation de la mémoire qui permettrait une démethylation et activation de régulateurs positifs de la mémoire et/ou la méthylation et la répression de régulateurs négatifs de la formation de la mémoire après apprentissage.

Vor kurzem wurde eine neue DNA-Modifikation, 5-hydroxymethylcytosine (5hmC), entdeckt, welche durch Oxydierung von 5-methylcytosine (5mC) durch TET1 Enzyme entsteht und als einen Zwischenprodukt der DNA-demethylierung erachtet wird. Vorbereitende Analysen zu 5hmC zeigen, dass es im Gehirn vorkommt und vor allem in Hirnregionen entscheidend für Lernen und Verankerung angereichert ist. Dies lässt auf eine potentielle Rolle von 5hmC in der Etablierung und Verankerung des Furcht-Gedächtnisses in erwachsenen Mäusen schliessen. Um diese Hypothese zu untersuchen, wurde eine kombinierte Analyse des Profils von cytosine Hydroxymethylierung und Methylierung am Promoter von creb1 und pp1 im Hippocampus durchgeführt. Bei diesen Genen handelt es sich jeweils um, einen positiven und einen negativen Regulator des Gedächtnisses. Diese Studie erbringt preliminäre Daten, dass in Folge von Furcht-Konditionierung und Consolidation des Furcht Gedächtnisses das Niveau von 5hmC am creb1 Promoter erhöht wird, während am pp1 Promoter das Niveau von 5mC erhöht wird. Diese anfänglichen Ergebnisse legen eine dynamische Regelung von 5hmC im Hippocampus während Gedächtnisverankerung nahe. Im Zuge dessen, lassen sie vermuten, dass es sich dabei um einen Mechanismus zur Demethylierung und damit Aktivierung von positiven Regulatoren des Gedächtnisses handelt. Auf der anderen Seite könnten negative Regulatoren der Gedächtnisbildung durch erhöhte Methylierung vermindert werden.