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Cortical DNA methylation maintains remote memory

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A behavioral memory's lifetime represents multiple molecular lifetimes, suggesting the necessity for a self-perpetuating signal. One candidate is DNA methylation, a transcriptional repression mechanism that maintains cellular memory throughout development. We found that persistent, gene-specific cortical hypermethylation was induced in rats by a single, hippocampus-dependent associative learning experience and pharmacologic inhibition of methylation 1 month after learning disrupted remote memory. We propose that the adult brain utilizes DNA methylation to preserve long-lasting memories.

In recent years, neuroscience has gained a relatively deep understanding of how memories are formed. In stark contrast is our limited understanding of how these same memories are maintained[1–4]. Previously, we found that hippocampal DNA methylation is critical for memory formation[5]. However, these hippocampal changes are transient, returning to basal levels in 1 day of learning. We used contextual fear conditioning to explore the possibility that cortical DNA methylation supports long-lasting memories. Contextual fear memory persists in rodents for many months, during which time the memory transitions from 'recent' to 'remote'. This change is thought to represent system consolidation, in which control over the memory shifts from the hippocampus (HPC) to a long-term dependence on the dorsomedial prefrontal cortex (dmPFC; anterior cingulate (ACC) and prelimbic cortices)[6,7].

We began by examining cortical DNA methylation of three memory-associated genes with large, GC-rich CpG islands (*Egr1*, *reelin* (*Reln*) and *calcineurin* (*CaN*, also known as *Ppp3ca*)) using methylated DNA immunoprecipitation (MeDIP)[8] (**Supplementary Methods**). A subgroup of rats were tested to confirm the presence of fear memory at 7 d (context, 4.3% ± 2.1; shock, 2.3% ± 0.8; context and shock, 41.1% ± 3.2; $F_{2,14} = 91.35$, $P \leq 0.001$).

The immediate early gene *Egr1* was demethylated in all groups at all of the time points that we examined (**Supplementary Fig. 1**). Thus, environmental stimuli are broadly capable of altering the methylation state of *Egr1* in the dmPFC. In contrast, *Reln*, a positive regulator of memory[9], was hypermethylated in trained rats 1 h after training. The amount of hypermethylation in the trained rats was reduced at later time points, (**Supplementary Fig. 1**). Methylation of the phosphatase and memory suppressor *CaN*[10] was not affected shortly after training. However, *CaN* was hypermethylated within 1 d of training in trained rats (**Fig. 1a**). We focused on *CaN* as a proxy for persistent methylation that is probably present on many genes after learning. Moreover, *CaN*'s pattern of methylation suggests that the HPC and dmPFC may communicate during the initial 24 h after learning as part of a system consolidation process that involves changes to cortical DNA methylation over time.

We next used bisulfite sequencing with subcloning to map the cytosine-specific methylation changes in *CaN* 7 d after training. We only found significant *CaN* methylation in the trained rats ($P \leq 0.05$; **Supplementary Fig. 2**). We then expanded our analysis, focusing on the naive and trained groups, to obtain a more detailed map. There was no evidence of single sequences with substantially increased methylation (**Fig. 1b**) and methylated CpGs were randomly distributed across the amplicon (**Fig. 1c**). In addition, the resulting single-allele sequences indicated very low levels of methylation in the amplified region (**Fig. 1b**,**c**), consistent with reports of typical methylation levels in CpG islands[11] and memory's sparse encoding pattern. Analysis of the additional clones confirmed that *CaN* hypermethylation was specific to the context plus shock group (naive, 0.08 ± 0.01; context plus shock, 0.25 ± 0.04; $F_{1,7} = 12.91$, $P \leq 0.05$).

To determine whether the lasting cortical methylation reflects associative learning, we gave rats pretraining injections of the NMDA receptor antagonist MK-801. A subgroup of rats, tested 7 d post-training, confirmed the ability of NMDA receptor antagonism to interfere with acquisition of a fear memory[12] (vehicle, 59.4% ± 5.3; MK-801, 19.0% ± 2.7; $F_{1,13} = 50.22$, $P \leq 0.001$). MK-801 also prevented the dmPFC *CaN* (vehicle, 1.24 ± 0.2; MK-801, 0.64 ± 0.1; $F_{1,13} = 50.22$, $P \leq 0.001$) and *Reln* hypermethylation at 7 d, without affecting *Egr1* hypermethylation (**Supplementary Fig. 3**), indicating that *CaN* and *Reln* hypermethylation is a specific response to associative environmental signals.

A previous study investigated what effect inactivation of the ACC, a subregion of the dmPFC, has on fear memory retrieval at various post-training time points[6]. ACC inactivation at 18 and 36 d (remote memory), but not 1 or 3 d post-training (recent memory), interfered with retrieval. This suggests that system consolidation occurs between 3 and 18 d and further suggests that the cortical DNA methylation events that we observed during the first week post-training (**Fig. 1**) are appropriately timed to participate in the initial incorporation of a memory trace in the cortex. We infused the NMDA receptor antagonist D(–)-2-amino-5-phosphonovaleric acid (AP5) directly into the dorsal HPC (CA1) immediately before training. AP5 not only interfered with learning (vehicle, 51.5% ± 11.7; AP5, 12.3% ± 6.1; $F_{1,9} = 9.73$, $P \leq 0.05$) but also blocked *CaN* (vehicle, 2.11 ± 0.2; AP5, 0.39 ± 0.1; $F_{1,13} = 50.22$, $P \leq 0.001$) and *Reln* (**Supplementary Fig. 3**) methylation in the dmPFC 7 d after training, indicating that

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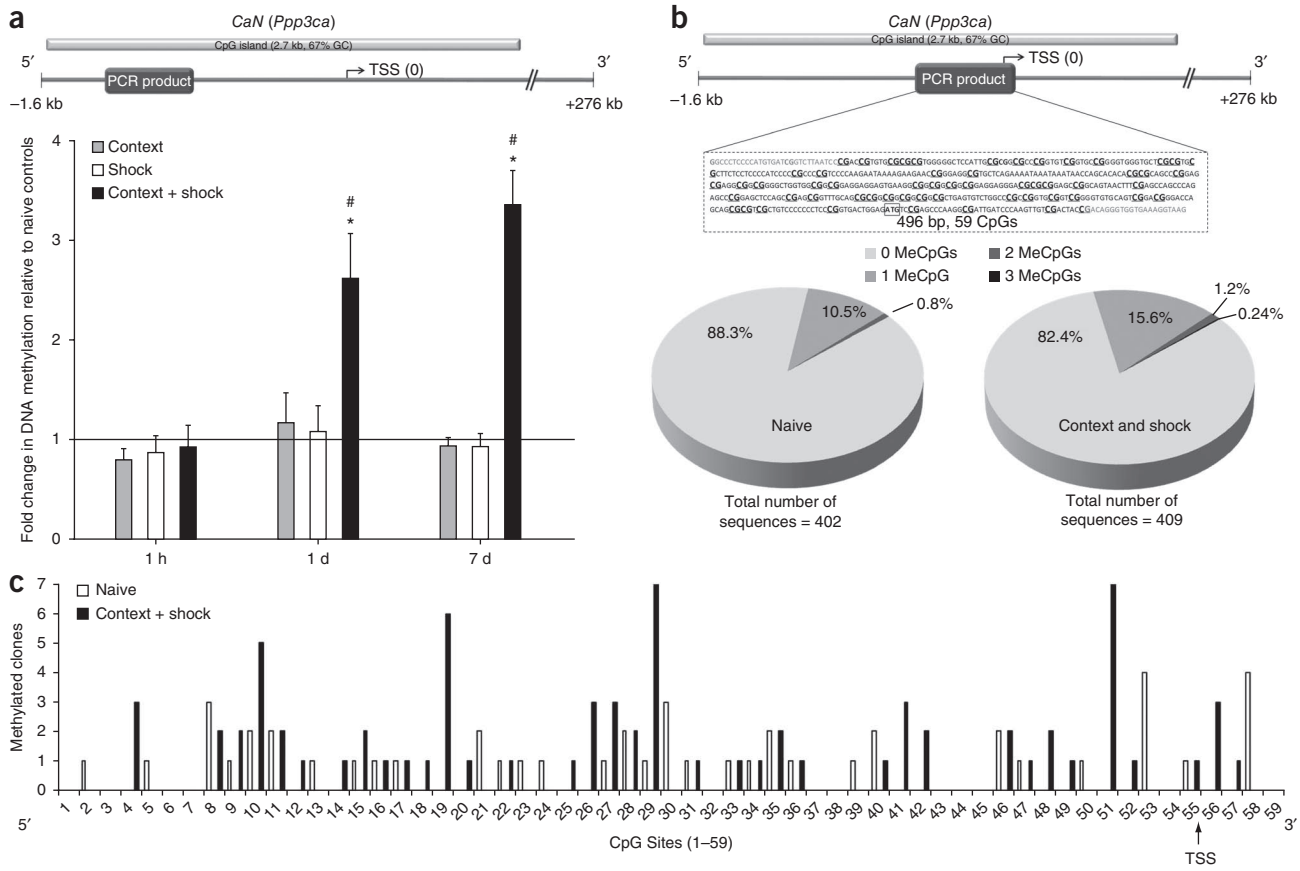

Figure 1 Learning induces persistent DNA methylation of *CaN* in the prefrontal cortex. (**a**) *CaN* CpG island analyzed and primer location. *CaN* was hypermethylated in context and shock rats at 1 and 7 d (no retrieval test; 1 h, $F_{3,18} = 0.27$; 1 d, $F_{3,19} = 5.73$; 7 d, $F_{3,25} = 33.52$; *post hoc* test, $^{*}P \leq 0.05$). *CaN*'s hypermethylation was significantly greater at 1 and 7 d ($F_{2,20} = 13.96$, $^{\#}P \leq 0.01$). See **Supplementary Table 1** for *n* values. TSS, transcription start site. (**b**) Amplicon analyzed by bisulfite sequencing and subcloning at 7 d. Charts depict the number of alleles with 0, 1, 2 or 3 methylated CpGs in the amplicon. (**c**) Single allele cytosine methylation for the 59 CpGs located in the amplicon at 7 d ($n = 4$ per group). See **Supplementary Table 2** for primer sequences. Error bars indicate s.e.m.

a single hippocampus-dependent learning experience is sufficient to drive lasting, gene-specific methylation changes in the cortex. These findings present a tangible marker that can be used for a learning-induced dialog between the HPC and dmPFC.

To support memory persistence, cortical DNA methylation would need to be long lasting. Therefore, we examined the persistence of fear memory (context, 0% ± 0; shock, 1.3% ± 1.3; context plus shock, 46.1% ± 8.3; $F_{2,15} = 29.35$, $P \leq 0.001$) and the methylation status of these genes 30 d after training. We observed robust methylation of *CaN* in context plus shock rats (**Fig. 2a** and **Supplementary Fig. 4**) well into the time period in which memories are thought to become dependent on this region. In addition, the rapid methylation that we observed in the HPC[5] versus sustained methylation in the dmPFC is consistent with system consolidation[7]. In accordance with DNA methylation's role as a transcriptional repressor, we found that *CaN* mRNA (**Fig. 2b** and **Supplementary Fig. 4**) and protein (**Fig. 2c**) were specifically reduced in context plus shock rats following retrieval of the 30-d-old memory.

Finally, to determine whether DNA methylation is necessary for the maintenance of a remote memory, we inhibited the enzymes responsible for introducing and maintaining cytosine methylation, DNA methyltransferases (DNMT), 30 d after training using 5-azadeoxycytidine (5-aza) or zebularine (zeb). Rats that received intra-ACC infusions of DNMT inhibitors failed to display normal memory (**Fig. 3a** and **Supplementary Fig. 5**), indicating that DNA methylation in the ACC is critical for remote memory stability. However, outside of the CNS, both compounds require DNA replication. As their mechanism is currently unclear in the CNS, we confirmed that a direct DNMT inhibitor, RG108, also interfered with remote memory (**Fig. 3a**). In addition, DNMT inhibitors

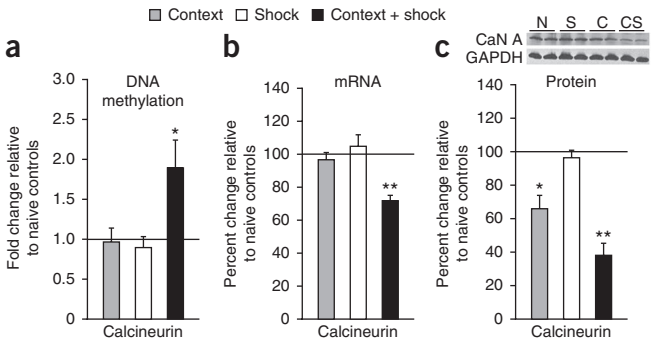

Figure 2 Cortical DNA methylation persists for at least 30 d. (**a**) *CaN* was still hypermethylated 30 d after training ($F_{3,19} = 4.77$; $^{*}P \leq 0.05$). (**b**) *CaN* transcript was decreased in context plus shock at 30 d ($t_5 = -8.36$, $^{**}P \leq 0.001$, $F_{2,15} = 12.72$). (**c**) CaN protein levels were decreased in context plus shock 2 h after retrieval test ($F_{3,30} = 24.31$). See **Supplementary Table 3** for *n* values. Error bars indicate s.e.m.

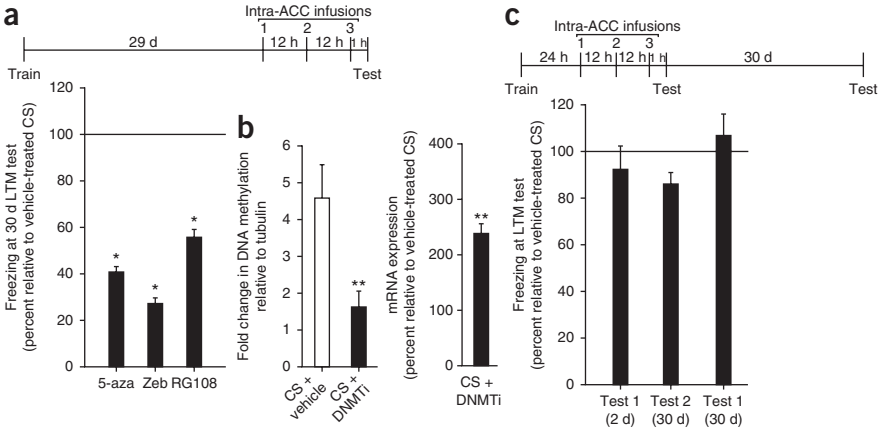

Figure 3 Cortical DNA methylation is required for remote memory. (**a**) Intra-ACC infusions of 5-azadeoxycytidine (5-aza, $n = 14$ and 13, $F_{1,26} = 11.48$), zebularine (zeb, $n = 7$ and 7, $F_{1,13} = 21.07$) or RG108 ($n = 10$ and 10, $F_{1,19} = 5.17$) 30 d after training disrupted remote fear memory ($^{*}P \leq 0.05$). (**b**) DNMT inhibitors (DNMTi) interfered with context plus shock (CS)-induced *CaN* methylation ($n = 6$ and 6, $F_{1,11} = 8.96$) and normalized *CaN* transcript levels ($t_8 = 8.34$, $^{**}P \leq 0.001$). (**c**) Intra-ACC infusions of DNMT inhibitors 1 d after training had no effect on fear memory ($n = 7$ and 8, $F_{1,14} = 0.81$, $P > 0.05$). The same rats also expressed normal fear memory 30 d later ($F_{1,12} = 0.26$, $P > 0.05$). Experiment was repeated in absence of a test at 2 d to confirm lack of damage to ACC. Again, infusions had no effect on fear memory ($n = 7$ and 8, $F_{1,14} = 0.81$, $P > 0.05$). Error bars indicate s.e.m.

interfered with the hypermethylation and reduced transcription of *CaN* (**Fig. 3b**), confirming its specific effect on DNA methylation.

Intra-ACC infusions of a DNMT inhibitor may produce state-dependent effects or affect the health of cells. To address these potential confounds, we performed a control experiment in which rats received the same infusions 24 h after training, before the memory had become reliant on the ACC. Both rats treated with vehicle and rats treated with DNMT inhibitors had normal fear memory, indicating that DNMT inhibitors do not interfere with a rat's physical ability to display a fear memory (**Fig. 3c**). Both groups also had normal memory 30 d post-training (**Fig. 3c**), indicating that the ACC is sufficiently healthy to support a memory, such that normal transfer is likely to have occurred during the intervening weeks. These data also suggest that any effect of these drugs on baseline methylation does not preclude the subsequent formation of a remote memory.

Our results indicate that methylation helps to maintain memories and may serve as a marker of the memory trace. This introduces additional, important questions. For instance, does methylation alter a neuron's basal state, thus altering its response to future stimuli? An example of this might be lowering a neuron's firing threshold to enable incorporation into the existing cortical network, which transcriptional repression of *CaN* may accomplish, given its importance in long-term depression[13] and interference with long-term potentiation[10]. Another possibility is that synaptic proteins and signaling pathways downstream of cellular activation utilize methylation as a mechanism to self-perpetuate through the regulation of their own transcription rate. For example, persistent changes in methylation may support ongoing synthesis of proteins that support input-specific changes to the synapse[14,15]. These possibilities need not be mutually exclusive. Regardless of the answers to these questions, our findings indicate that cortical DNA methylation is triggered by a learning experience and is a perpetuating signal used by the brain to help preserve remote memories. In combination with our previous work[5], these results suggest that DNA methylation provides the brain with a mechanism that enables it to be dynamic during memory formation but also stable thereafter to maintain those memories.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

C.A.M. and J.D.S. conceived of the project. C.A.M., G.R. and J.D.S. designed the experiments. G.R. contributed to assay development. C.A.M., C.F.G., J.A.W., R.R.P., I.M.R., A.H., M.D.R. and C.R.Y. performed the experiments. C.A.M. wrote the manuscript. G.R. and J.D.S. edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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