The formation of fear extinction memory requires the accumulation of N6-methyl-2'-deoxyadenosine in DNA

Xiang Li[1,2♭], Qiongyi Zhao[1], Wei Wei[1], Quan Lin[5], Christophe Magnan[3], Michael Emami[2], Luis E. Wearick da Silva[4], Thiago W. Viola[4], Paul Marshall[1], Jordan Edmunds[2], Sara Nainar[6], Cathrine Broberg Vågbø[7], Laura Leighton[1], Esmi Zajaczkowski[1], Ke Ke[2], Rodrigo Grassi-Oliveira[4], Magnar Bjørås[7], Pierre F. Baldi[3], Robert C. Spitale[6] and Timothy W. Bredy[1,2♭]

[1]Queensland Brain Institute, The University of Queensland, Brisbane, QLD, Australia 4072; [2]Department of Neurobiology and Behavior and Center for the Neurobiology of Learning and Memory, University of California Irvine, Irvine, CA, USA 92697; [3]Department of Computer Science and Institute for Genomics and Bioinformatics, University of California Irvine, Irvine, CA 92697; [4]Biomedical Research Institute, Pontifical Catholic University of Rio Grande do Sul, Porto Alege, Brazil; [5]Intellectual Development and Disabilities Research Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, USA; [6]Department of Pharmaceutical Sciences, University of California Irvine, Irvine, CA, USA 92697; [7]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway.

♭Please send correspondence to:

Timothy W. Bredy, Ph.D.
Email: t.bredy@uq.edu.au

Or

Xiang Li, Ph.D.
Email: x.li12@uq.edu.au

Keywords: m6dA, BDNF, DNA modification, extinction, memory, prefrontal cortex

Acknowledgements: The authors gratefully acknowledge grant support from the NIH (5R01MH105398-TWB and PB), and NIGMS (1DP2GM119164-01-RCS), the NHMRC (APP1033127-TWB), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-CsF-400850/2014-1-RGO), and the Research Council of Norway (FRIMEDBIO grant 32222-MB). XL is supported by postgraduate scholarships from the University of Queensland and the ANZ trustees Queensland for medical research. The authors would also like to thank Ms. Rowan Tweedale for helpful editing of the manuscript, and especially Sunil Gandhi for comments and discussion.

Here we report that the recently identified DNA modification N6-methyl-2'-deoxyadenosine (m6dA) is dynamically regulated in post-mitotic neurons and accumulates within promoters and genomic coding sequences of neurons activated by fear extinction learning in adult C57/Bl6 mice. The deposition of m6dA leads to an open chromatin state as well as the recruitment of the activating transcription factor Yin-Yang 1 and RNA polymerase II, which are critical for activity-induced brain-derived neurotrophic factor gene expression and required for the formation of fear extinction memory. These findings expand the scope of DNA modifications in the adult brain and highlight the discovery of DNA m6dA as novel neuroepigenetic mechanism associated with learning and memory.

Introduction

In recent years, our understanding of neural plasticity, learning, and memory has been advanced by the demonstration that various epigenetic processes are involved in the regulation of experience-dependent gene expression in the adult brain[1]. DNA methylation, once considered static and restricted to directing cellular lineage specificity during early development, is now recognized as being highly dynamic and reversible across the lifespan[2-4]. Although more than 20 DNA modifications have been identified[5], nearly all research aimed at elucidating the role of these chemical modifications in the brain has focused on either 5-methylcytosine (5mC) or the recently rediscovered 5-hydroxymethycytosine (5hmC), which is a functionally distinct oxidative derivative of 5mC[6-8]. 5mC and 5hmC are highly prevalent in neurons relative to other cell types[6,9] and both modifications are regulated in response to learning[10-12].

Apart from 5mC and 5hmC, N6-methyl-2'-deoxyadenosine (m6dA) is the most abundant DNA modification in prokaryotes. In bacteria, m6dA regulates replication, transcription, and transposition[13]. Until recently, m6dA had only been detected in unicellular eukaryotes[14,15]; however, due to significant improvements in mass spectrometry and sequencing technology, m6dA has recently been shown to accumulate in a variety of eukaryotic genomes. For example, in *Chlamydomonas reindardtii,* m6dA is deposited at the transcription start site[16], and in *Drosophila*, the level of m6dA increases across development and is enriched within transposable elements[17]. Furthermore, m6dA appears to be involved in reproductive viability in *Caenorhdoditis elegans*[18]. These observations have led to speculation that m6dA may play a role in the regulation of gene expression. Although m6dA is present in the genome of vertebrates[19-21], whether it is dynamic in post-mitotic neurons and associated with learning and memory has yet to be determined.

Results

m6dA is dynamically regulated by in post-mitotic neurons by neuronal activity. Given that the enrichment of chemical modifications in neuronal DNA confers control over gene expression programs related to cellular identity during early development and in differentiated neurons, and that m6dA is associated with transcription in lower eukaryotes[16], we hypothesized that m6dA may also be fundamental for governing experience-dependent gene expression within the mammalian genome. We therefore

investigated the role of m6dA within neurons and sought to elucidate whether it is involved in the regulation of activity-induced gene expression (Suppl. Fig. 1A). We first employed a gel shift assay using genomic DNA derived from primary cortical neurons treated with the restriction enzyme DpnI, which cuts DNA specifically at methylated adenines predominantly in a GATC sequence context, revealing evidence in favor of m6dA as a modified base in neuronal DNA (Suppl. Fig. 1B)[16]. To verify these findings, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to quantify the global level of m6dA within cortical neurons. The occurrence of m6dA was estimated to be ~46 per 10^6 dNs (~280,000 m6dA) across the neuronal genome (Suppl. Fig. 1C). We next performed a dot blot assay in order to determine whether m6dA is dynamic in neuronal DNA. A standard KCl-induced depolarization protocol was used to induce neuronal activity *in vitro*[22] revealing a significant global accumulation of m6dA upon stimulation (Suppl. Fig. 1D). Together, these data strongly suggest that m6dA is both a dynamic and prevalent base modification in differentiated neurons. This is in stark contrast to a recent report that m6dA is not present at detectable levels in embryonic stem cells and whole brain[23]**.** The relative abundance and distribution of m6dA in the mammalian genome may therefore be cell-type specific, with greater accumulation in activated post-mitotic neurons than in other cell types.

m6dA accumulates in an experience-dependent manner in the adult brain. The inhibition of learned fear is an evolutionarily conserved behavioral adaptation that is essential for survival. This process, known as fear extinction, involves rapid reversal of previously learned contingencies, and depends on rapid adaptive gene expression in various brain regions. The paradigm of fear extinction has long been recognized as an invaluable tool for investigating the neural mechanisms of emotional learning and memory, and the important contribution of the ILPFC to extinction has been demonstrated[24]. A variety of epigenetic mechanisms in the ILPFC have been implicated in fear extinction[12,25,26]; therefore, this behavioral model serves as a robust platform to interrogate the role of epigenetic mechanisms in a critically important memory process.

Using activity regulated cytoskeleton associated protein (Arc) and a neuronal nuclear marker (NeuN) as tags for whole-cell fluorescence-activated cell sorting (FACS), we enriched for post-mitotic neurons

selectively activated by extinction learning from mice that had been recently trained on a fear extinction task (Fig. 1A, Suppl. Fig. 2). A well-established DpnI-seq approach[27] was then applied to map the genome-wide extinction learning-induced accumulation of m6dA at base resolution. In line with previous reports[28,29], we found that DpnI cleaves a dominant fraction of methylated GATC sites (Fig. 1B), with a relatively low abundance of m6dA within intergenic regions (Suppl. Fig 3). An extinction learning-induced accumulation of m6dA was highly prevalent in the promoter and coding regions (CDS). Notably, there was a characteristic drop in m6dA at -2bp from the transcription start site (TSS) and -1bp from the transcription end site (TES) (Fig. 1C). Previous studies found that the accumulation of m6dA primarily occurs in the promoter and around the TSS[16,17,27]. By quantifying the abundance of m6dA, we demonstrated an enormous increase in the accumulation of m6dA at a site +1bp downstream of the TSS (Fig. 1D). We also observed a highly significant increase in m6dA deposition +4bp from the start codon, followed by an overall increase in the m6dA accumulation across the CDS (Fig. 1E).

A comparison between fear-conditioned mice without extinction (FC-No EXT) and fear-conditioned mice with extinction (EXT) revealed significant differences in the experience-dependent accumulation of m6dA (Fig. 1F). From a total of 2839 differentially m6dA methylated sites, 1772 GATC sites were specific to EXT mice. A gene ontology (GO) analysis revealed that the most significant cluster specific to EXT was for “synapse” (Fig. 1G). Six of the top synapse-related genes that exhibited a significant accumulation of m6dA in response to extinction training (Fig. 1H), including brain-derived neurotrophic factor (bdnf), have previously been shown to be related to neural plasticity, learning and memory[25,30].

Bdnf is the most widely expressed inducible neurotrophin in the central nervous system[31], and is directly involved in extinction-related learning and memory[30]. In the adult brain, 5mC within bdnf gene promoters is altered by experience[32], and this regulation appears to be necessary for the regulation of gene expression underlying remote memory[10]. The bdnf locus comprises at least eight homologous noncoding exons that contribute to alternate 5’untranslated regions (UTRs), and a ninth that contributes a protein coding sequence and 3’UTR[33,34]. The complex structure of this locus has led to idea that bdnf expression may be driven by DNA modifications that guide distinct sets of transcription factor complexes to initiate the transcription of the various isoforms[35], all of which could be important for learning and memory formation.

This is supported by the fact that exon IV is highly activity-dependent and plays a direct role in the formation of fear extinction memory[25,36]. There was a highly specific accumulation of m6dA at a GATC site immediately downstream of the TSS of the BDNF P4 promoter in fear extinction trained mice (Suppl. Fig. 4). Surprisingly, this is the only GATC site found within 500bp around the TSS of bdnf exon IV further suggesting a high level of selectivity with respect to where and when m6dA dynamically accumulates in the genome in response to experience.

N6amt1 mRNA expression is associated with neuronal activation. N6 adenine-specific DNA methyltransferase 1 (N6amt1) was originally described as a mammalian ortholog of the yeast adenine methyltransferase MTQ2. Homologs of N6amt1 have been shown to methylate N6-adenine in bacterial DNA[37] and a previous study identified mammalian N6amt1 as a glutamine-specific protein methyltransferase[38]. Whether the same process occurs in mammalian DNA remains unclear[39]. Importantly, according to the Allen Brain Atlas, N6amt1 is highly expressed in the mouse neocortex (http://mouse.brain-map.org/experiment/show?id=1234), which is in accordance with the findings of Ratel et al[40]. The expression of N6amt2, which shares a highly-conserved methyltransferase domain, is also expressed in the mouse brain (http://mouse.brain-map.org/experiment/show?id=69837159). In order to elucidate the underlying mechanism by which m6dA accumulates in the mammalian genome, N6amt1 and N6amt2 were selected for an analysis of their potential role in regulating the deposition of m6dA on neuronal DNA. N6amt1 exhibited a time-dependent increase in mRNA expression in primary cortical neurons in response to KCl-induced depolarization (Suppl. Fig 5A, t-test, t_6=4.14, **p<.01), whereas there was no effect on N6amt2 (Suppl. Fig. 5B). These findings suggest that the gene encoding N6amt1, but not N6amt2, is activity-induced in post-mitotic neurons. We next asked whether N6amt1 was functionally involved in regulating the activity-induced deposition of m6dA in post-mitotic neurons.

Activity-induced N6amt1-mediated accumulation of m6dA promotes BDNF exon IV expression *in vitro*. Chromatin immunoprecipitation analysis revealed an increase in the occupancy of N6amt1 at the BDNF P4 promoter (Fig. 2A, t-test, t_6=2.77, *p<.05), but not N6amt2 (Fig. 2B, t-test, t_4=3.142, *p<.05), This

was accompanied by a significant increase in the deposition of m6dA at the same locus (Fig. 2C, t-test, t_4=3.934, **p<.01). In order to more precisely define the accumulation of m6dA, the Dpn1 restriction enzyme digestion approach was again applied. KCl-induced depolarization led to an increase of m6dA at the GATC site (Fig 2D. t-test, t_4=2.76, *p<.05). Adjacent to this site, there is a consensus sequence for the activating transcription factor Yin-Yang (YY1). Moreover, it has previously been shown that the accumulation of m6dA is associated with an active TSS[16]. This suggests that an increase in m6dA may lead to the establishment of an active chromatin environment. Indeed, neuronal stimulation led to increased occupancy of the active chromatin mark H3K4^{me3} (Fig. 2E, t-test, t_{14}=2.59, *p<.05), as well as increased binding of YY1 proximal to the m6dA-modified GATC site (Fig. 2F, t-test, t_4=5.58, **p<.01). There was also a concomitant increase in the presence of RNA polymerase (Pol) II at this site (Fig. 2G, t-test, t_4=4.04, **p<.01), which is strongly predictive of transcriptional elongation. The activity-induced changes in N6amt1 occupancy, m6dA accumulation and related changes in the local chromatin landscape and transcriptional machinery correlated with the induction of bdnf exon IV mRNA expression (Fig. 2H, t-test, t_6=16.08, ***p<.001). Importantly, at a distal GATC site located within the bdnf P4 promoter 1000bp upstream of TSS, the observed m6dA deposition and associated changes did not occur (Suppl. Fig. S6A-E).

In primary cortical neurons infected with a lentiviral vector encoding N6amt1 shRNA, the observed activity-dependent N6amt1 occupancy and m6dA deposition at the exon IV locus was eliminated (Suppl. Fig. S7A-B). This effect correlated with reduced H3K4^{me3}, decreased recruitment of YY1 and diminished Pol II occupancy at the predicted GATC site within the P4 promoter (Suppl. Fig. S7C-E). These changes in the local chromatin landscape, and impaired recruitment of transcription factors led to reduce bdnf exon IV mRNA expression (Suppl. Fig S7F). Together, these data demonstrate that an N6amt1-associated accumulation of m6dA in DNA promotes bdnf exon IV expression in response to neural activation. Furthermore, our findings suggest that the activity-dependent deposition of m6dA by N6amt1 may occur in a tightly regulated, and time-dependent, manner through locus-specific chromatin modification and the subsequent recruitment of activating transcription machinery (Fig. 2I).

Extinction learning-induced N6amt1-mediated accumulation of m6dA promotes bdnf exon IV expression *in vivo*. To determine whether the effects observed in primary cortical neurons are functionally relevant in the adult brain, we examined the role of m6dA in regulating extinction learning-induced bdnf exon IV mRNA expression within the infralimbic prefrontal cortex (ILPFC). Similar to the effect of KCl-induced depolarization on m6dA and correlated gene expression *in vitro*, extinction training led to a significant increase in N6amt1 mRNA expression in the ILPFC (Suppl. Fig. S8A, t-test, t_6=3.64, **p<.01), again with no detectable change in N6amt2 (Suppl. Fig. S8B). In contrast to the ILPFC, hippocampal N6amt1 and N6amt2 mRNA showed no change in expression in response to behavioral training (Suppl. Fig. S8C-D). Extinction training also led to an increase in N6amt1 occupancy relative to that in mice that had been fear conditioned and only exposed to a novel context (FC-No EXT) (Fig. 3A, t-test, t_{10}=1.87, *p<.05), and this was accompanied by increased deposition of m6dA in the EXT mice (Fig. 3B, t-test, t_{10}=2.81, *p<.05). The data also revealed a significant increase in $H3K4^{me3}$ occupancy (Fig. 3C, t-test, t_{14}=2.57, *p<.05), an increase in the recruitment of YY1 (Fig. 3D, t-test, t_{10}=1.89, *p<.05), and increased Pol II occupancy (Fig. 3E, t-test, t_{10}=2.87, **p<.01). Finally, in agreement with the known role of bdnf in fear extinction, there was a significant increase in bdnf exon IV mRNA expression in the ILPFC in response to fear extinction training (Fig. 3F, t-test, t_{14}=2.75, **p<.01). Similar to the observations in primary cortical neurons *in vitro*, m6dA deposition and the associated changes in chromatin structure following extinction training were not observed at the distal GATC site located within the BDNF P4 promoter (Suppl. Fig. S9A-F).

N6amt1-mediated accumulation of m6dA is associated with gene expression and fear extinction memory formation. Having established a relationship between the extinction learning-induced accumulation of m6dA and the regulation of bdnf exon IV mRNA expression *in vivo*, we next investigated whether lentiviral-mediated knockdown of N6amt1 in the ILPFC affects the formation of fear extinction memory (Fig. 4A). We first validated the efficiency of the knockdown construct *in vivo*, which showed excellent transfection efficiency and a reliable decrease in N6amt1 mRNA expression when infused directly into the ILPFC prior to behavioral training (Suppl. Fig. S10A-B). There was no effect of N6amt1 shRNA on

within-session performance during the first 10 conditioned stimulus exposures during fear extinction training (Fig. 4B-C), and there was no effect of N6amt1 shRNA on fear expression in mice that had been fear conditioned and exposed to a novel context without extinction training (Fig. 4D-left, pre-CS). However, there was a highly significant impairment in fear extinction memory in mice that had been extinction trained in the presence of N6amt1 shRNA (Fig. 4D-right, n=8/group, two-way ANOVA $F_{1,28}$= 9.18, p<.01; Bonferroni's posthoc test; EXT scrambled vs. EXT shRNA, ***p<.001). Infusion of N6amt1 shRNA into the prelimbic region of the prefrontal cortex (PLPFC), a brain region immediately dorsal to the ILPFC that is not required for the acquisition or expression of fear extinction memory had no effect (Fig. 4E-G). These data demonstrate a critical role for the N6amt1-mediated accumulation of m6dA in the ILPFC in regulating the formation of fear extinction memory.

With respect to the epigenetic landscape and transcriptional machinery surrounding the bdnf P4 promoter *in vivo*, knockdown of N6amt1 prevented the extinction learning-induced increase in N6amt1 occupancy (Fig. 5A, two-way ANOVA $F_{1,12}$ = 6.36, p<.05; Bonferroni's posthoc test; EXT scrambled vs. EXT N6amt1 shRNA, *p<.05) and the accumulation of m6dA (Fig. 5B, two-way ANOVA $F_{1,12}$ = 2.87, p<.05; Bonferroni's posthoc test; EXT scrambled vs. EXT N6amt1 shRNA, *p<.05). N6amt1 knockdown also blocked the extinction-learning induced increase in occupancy of $H3K4^{me3}$ (Fig. 5C, two-way ANOVA $F_{1,12}$ = 7.59, p<.05; Bonferroni's posthoc test; EXT scrambled vs. EXT N6amt1 shRNA, *p<.05), YY1 (Fig. 5D, two-way ANOVA $F_{1,12}$ = 14.87, p<.01; Bonferroni's posthoc test; EXT scrambled vs. EXT N6amt1 shRNA, **p<.01), and Pol II occupancy at the BDNF P4 promoter (Fig. 5E, two-way ANOVA $F_{1,12}$ = 75.13, p<.0001; Bonferroni's posthoc test; EXT scrambled vs. EXT N6amt1 shRNA, ***p<.001). Finally, similar to our *in vitro* findings, N6amt1 knockdown blocked the effect of extinction training on bdnf exon IV mRNA expression (Fig. 5F, two-way ANOVA $F_{1,12}$ = 12.57, p<.01; Bonferroni's posthoc test; FC- No EXT scrambled vs. Ext scrambled, **p<.01). Taken together, our findings suggest that a dynamic, learning-induced, accumulation of m6dA in the adult ILPFC is necessary for experience-dependent bdnf exon IV expression and the formation of extinction memory. A more generalized role for m6dA in other forms of learning and memory cannot yet be ruled out and future experiments should determine whether m6dA broadly serves as a mechanism of learning-dependent gene induction in the brain.

Discussion

Although more than 20 different base modifications are known to occur in DNA[5], only 5mC and 5hmC have been studied in any detail within the mammalian brain. Here we provide the first evidence that the dynamic accumulation of m6dA in post-mitotic neurons is associated with activity-induced gene expression and critically involved in the formation of fear extinction memory.

m6dA is a major DNA modification that is commonly found in bacterial DNA and lower eukaryotes[14,15,28,29,39,41]. However, its existence in higher eukaryotes is debated given previous observations of extremely low levels of m6dA in the mouse genome across different tissues[19,23,40]. In agreement with earlier findings, we could not detect m6dA in genomic DNA derived from mouse liver (Suppl. Fig. 1B). However, by using a combination of HPLC-MS/MS and an antibody-based dot blot assay, we were able to demonstrate a global induction of m6dA after neuronal activation. This was associated with an increase in the expression of the putative m6dA methyltransferase N6amt1 (Suppl. Fig. 4). These data suggest that post-mitotic neurons may employ m6dA as an epigenetic regulatory mechanism that is engaged specifically under activity-induced conditions.

We recently found that the pattern of 5mC within the adult brain differs in neurons and non-neuronal cells[42] and that 5hmC exhibits a dramatic redistribution in the adult ILPFC in response to extinction learning[12]. These lines of evidence suggest that learning-induced changes in DNA modification could be both dynamic and cell-type-specific. Our current findings indicate that the same functional characteristics may also apply to m6dA as it is evident that the accumulation of m6dA post-mitotic neurons relies on the activation state of post-mitotic neurons, a finding which may help to explain why previous studies were unable to detect m6dA in the mammalian genome. In accordance with this idea, we observed a distinct pattern of m6dA deposition within neurons that had been selectively activated by fear extinction learning compared to those neurons activated by context exposure (Fig. 1F).

In particular, it is noteworthy that the extinction learning-induced accumulation of m6dA was prominent not only around the TSS, but also along the CDS. These data suggest that m6dA may play an important role in initiating transcription by promoting an active chromatin state and the recruitment of Pol II, and contribute to the efficiency of Pol II read-through along the gene body. m6dA has been shown to

contribute to base flipping[43] and GATC methylation induces structural changes to DNA[44]. During elongation, these changes in DNA structure may maintain the transcription bubble by lowering the energetic barrier for the RNA polymerase active site[45,46]. Furthermore, m6dA has been shown to overlap with nucleosome-free regions[16], which also serve to facilitate transcription elongation[47]. Together with our observations, the evidence suggests an essential role for the deposition of m6dA within the CDS in promoting activity-induced transcriptional processes and that this is required for the underlying changes in gene expression that accompany the formation of fear extinction memory. Future studies will examine the direct relationship between the dynamic accumulation of m6dA and DNA structure states, and their influence on gene expression and on learning and memory.

YY1 initiates context-specific gene expression through interaction with Pol II[48-50], which is dependent on DNA methylation and changes in the local chromatin landscape[51-53]. In agreement with these findings, our data demonstrate that the activity-induced expression of bdnf exon IV both in primary cortical neurons, and within the ILPFC following behavioral training, is functionally related to an N6amt1-mediated increase in the accumulation of m6dA at the BDNF P4 promoter (Suppl. Fig. S6A-B and Fig. 3A-B). This is also associated with the presence of $H3K4^{me3}$, an epigenetic mark that reflects an active chromatin state (Suppl. Fig. S6C and Fig. 3C), and is accompanied by increased occupancy of the transcription factor YY1 and Pol II at the same locus (Suppl. Fig. S6D-E and Fig. 3D-E). Our findings demonstrate that the accumulation of m6dA surrounding the TSS of the BDNF P4 promoter drives activity-induced and experience-dependent exon IV mRNA expression, which is in agreement with recent findings on m6dA-mediated transcriptional activation in lower eukaryotes[16].

Other DNA modifications, including oxidative derivatives of 5mC, are found within gene promoters as well as gene bodies and have been shown to interact with Pol II to induce transient pausing of the Pol II elongation complex to promote gene expression[17,52,53]. This suggests that Pol II has the capacity to detect a variety of DNA modifications, including m6dA, and that this interaction may serve to fine-tune the rate of Pol II-mediated transcription. The high degree of specificity of m6dA accumulation at the specific GATC site proximal to the TSS in the BDNF P4 promoter further suggests that this particular DNA modification confers tight control over exon IV expression through regionally selective epigenetic regulation. Indeed, Pol II is

recruited to the P1 promoter in a spatiotemporally regulated manner, resulting in 'waves' of BDNF exon I mRNA expression[54]. Thus, the m6dA-mediated recruitment of YY1 and Pol II at activity-induced genes supporting memory formation may also provide a signal to sequester other epigenetic regulatory mechanisms in order to promote experience-dependent genomic metaplasticity, and to guide future patterns of learning-induced gene expression[3].

In summary, m6dA is dynamically regulated in the mammalian genome and its deposition is required for activity-induced BDNF expression and the formation of extinction memory. These findings significantly expand the scope of experience-dependent DNA modifications in the brain and indicate that the information-processing capacity of DNA in post-mitotic neurons is far more complex than current perspectives generally appreciate. We predict that a large number of functional modifications on *all four* canonical nucleobases remain to be discovered, and that it will be within the realm of cognition and memory that the impact of these novel epigenetic purveyors of genomic and behavioral diversity will prove most significant.

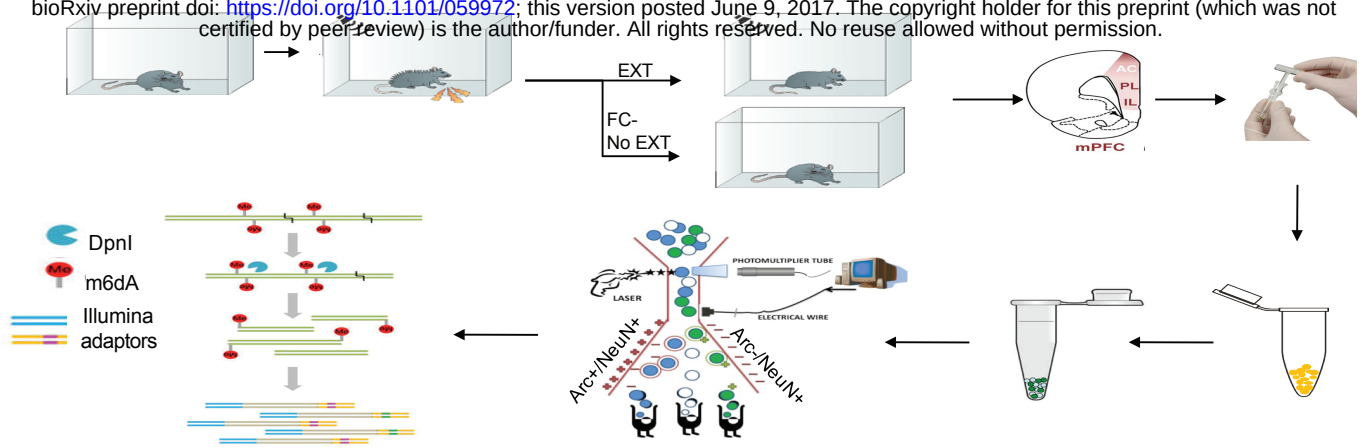
References

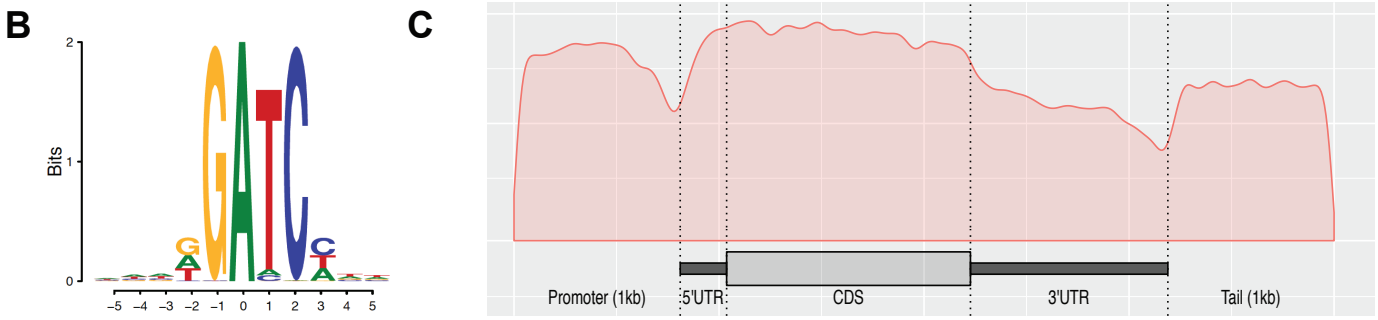
1. Marshall, P. & Bredy, T. W. Cognitive neuroepigenetics: the next evolution in our understanding of the molecular mechanisms underlying learning and memory? *NPJ Sci Learn* **1,** 16014 (2016).
2. Miller, C. A., Campbell, S. L. & Sweatt, J. D. DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiol Learn Mem* **89,** 599–603 (2008).
3. Baker-Andresen, D., Ratnu, V. S. & Bredy, T. W. Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioral adaptation. *Trends Neurosci* **36,** 3–13 (2013).
4. Gapp, K., Woldemichael, B. T., Bohacek, J. & Mansuy, I. M. Epigenetic regulation in neurodevelopment and neurodegenerative diseases. *Neuroscience* **264,** 99–111 (2014).
5. Korlach, J. & Turner, S. W. Going beyond five bases in DNA sequencing. *Curr. Opin. Struct. Biol.* **22,** 251–261 (2012).
6. Lister, R. *et al.* Global epigenomic reconfiguration during mammalian brain development. *Science* **341,** 1237905–1237905 (2013).
7. Guo, J. U., Su, Y., Zhong, C., Ming, G.-L. & Song, H. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* **145,** 423–434 (2011).
8. Shen, L. *et al.* Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. *Cell Stem Cell* **15,** 459–470 (2014).
9. Khare, T. *et al.* 5-hmC in the brain is abundant in synaptic genes and shows differences at the exon-intron boundary. *Nat. Struct. Mol. Biol.* **19,** 1037–1043 (2012).
10. Miller, C. A. *et al.* Cortical DNA methylation maintains remote memory. *Nature Neuroscience* **13,** 664–666 (2010).
11. Vanyushin, B. F., Mazin, A. L., Vasilyev, V. K. & Belozersky, A. N. The content of 5-methylcytosine in animal DNA: the species and tissue specificity. *Biochim. Biophys. Acta* **299,** 397–403 (1973).
12. Li, X. *et al.* Neocortical Tet3-mediated accumulation of 5-hydroxymethylcytosine promotes rapid behavioral adaptation. *Proc. Natl. Acad. Sci. U.S.A.* **111,** 7120–7125 (2014).
13. Iyer, L. M., Zhang, D. & Aravind, L. Adenine methylation in eukaryotes: Apprehending the complex evolutionary history and functional potential of an epigenetic modification. *BioEssays* **38,** 27–40 (2016).
14. Hattman, S., Kenny, C., Berger, L. & Pratt, K. Comparative study of DNA methylation in three unicellular eucaryotes. *J. Bacteriol.* **135,** 1156–1157 (1978).
15. Hattman, S. DNA-[adenine] methylation in lower eukaryotes. *Biochemistry (Moscow)* **70,** 550–558 (2005).
16. Fu, Y. *et al.* N6-methyldeoxyadenosine marks active transcription start sites in Chlamydomonas. *Cell* **161,** 879–892 (2015).
17. Zhang, G. *et al.* N6-methyladenine DNA modification in Drosophila. *Cell* **161,** 893–906 (2015).
18. Greer, E. L. *et al.* DNA methylation on N6-adenine in C. elegans. *Cell* **161,** 868–878 (2015).
19. Koziol, M. J. *et al.* Identification of methylated deoxyadenosines in vertebrates reveals diversity in DNA modifications. *Nat. Struct. Mol. Biol.* **23,** 24–30 (2015).
20. Wu, T. P. *et al.* DNA methylation on N6-adenine in mammalian embryonic stem cells. *Nature* **532,** 329–333 (2016).
21. Liu, J. *et al.* Abundant DNA 6mA methylation during early embryogenesis of zebrafish and pig. *Nat Comms* **7,** 13052 (2016).
22. Ataman, B. *et al.* Evolution of Osteocrin as an activity-regulated factor in the primate brain. *Nature* **539,** 242–247 (2016).
23. Schiffers, S. *et al.* Quantitative LC–MS provides no evidence for m6dA or m4dC in the genome of mouse embryonic stem cells and tissues. *Angew. Chem. Int. Ed. Engl.* **11,** 204 (2017).
24. Usheva, A. & Shenk, T. TATA-binding protein-independent initiation: YY1, TFIIB, and RNA polymerase II direct basal transcription on supercoiled template DNA. *Cell* **76,** 1115–1121 (1994).
25. Bredy, T. W. *et al.* Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn. Mem.* **14,** 268–276 (2007).
26. Wei, W. *et al.* p300/CBP-associated factor selectively regulates the extinction of conditioned fear. *J. Neurosci.* **32,** 11930–11941 (2012).

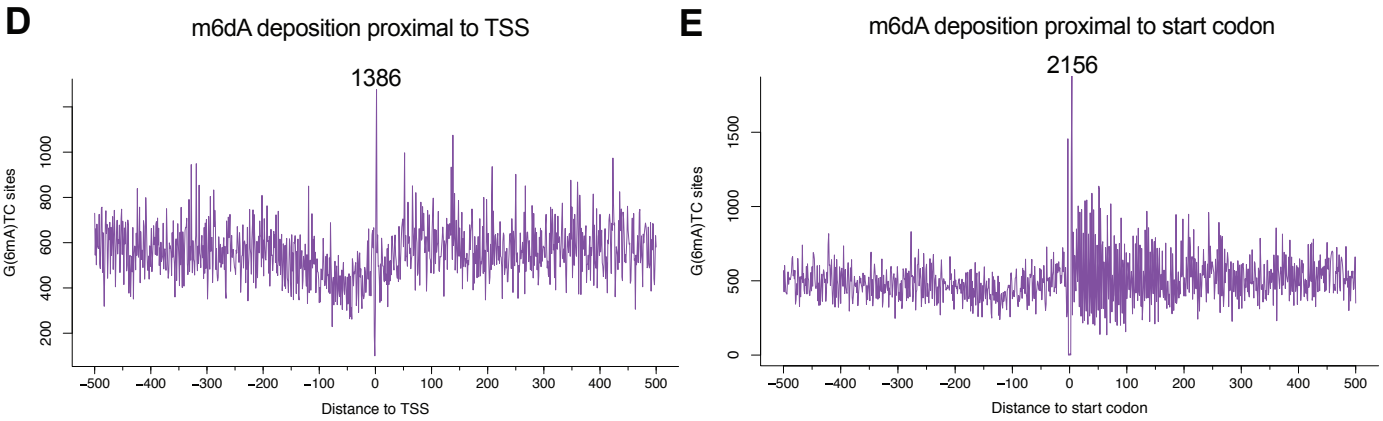
54 27. Luo, G.-Z. *et al.* Characterization of eukaryotic DNA N6-methyladenine by a highly sensitive
55 restriction enzyme-assisted sequencing. *Nat Comms* **7,** 11301 (2016).
56 28. Vovis, G. F. & Lacks, S. Complementary action of restriction enzymes endo R · DpnI and endo R ·
57 DpnII on bacteriophage f1 DNA. *J. Mol. Biol.* **115,** 525–538 (1977).
58 29. Lacks, S. & Greenberg, B. A deoxyribonuclease of Diplococcus pneumoniae specific for methylated
59 DNA. *J. Biol. Chem.* **250,** 4060–4066 (1975).
70 30. Peters, J., Kalivas, P. W. & Quirk, G. J. Extinction circuits for fear and addiction overlap in prefrontal
71 cortex. *Learn. Mem.* **16,** 279–288 (2009).
72 31. Ghosh, A., Carnahan, J. & Greenberg, M. E. Requirement for BDNF in activity-dependent survival of
73 cortical neurons. *Science* **263,** 1618–1623 (1994).
74 32. Lubin, F. D., Roth, T. L. & Sweatt, J. D. Epigenetic regulation of BDNF gene transcription in the
75 consolidation of fear memory. *J. Neurosci.* **28,** 10576–10586 (2008).
76 33. Aid, T., Kazantseva, A., Piirsoo, M., Palm, K. & Timmusk, T. Mouse and rat BDNF gene structure
77 and expression revisited. *J. Neurosci. Res.* **85,** 525–535 (2007).
78 34. West, A. E. & Orlando, V. Epigenetics in brain function. *Neuroscience* **264,** 1–3 (2014).
79 35. West, A. E. Biological functions of activity-dependent transcription revealed. *Neuron* **60,** 523–525
80 (2008).
81 36. Sakata, K. *et al.* Role of activity-dependent BDNF expression in hippocampal-prefrontal cortical
82 regulation of behavioral perseverance. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 15103–15108 (2013).
83 37. Stephens, C., Reisenauer, A., Wright, R. & Shapiro, L. A cell cycle-regulated bacterial DNA
84 methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1210–1214 (1996).
85 38. Liu, P. *et al.* Deficiency in a glutamine-specific methyltransferase for release factor causes mouse
86 embryonic lethality. *Mol. Cell. Biol.* **30,** 4245–4253 (2010).
87 39. Ratel, D., Ravanat, J.-L., Berger, F. & Wion, D. N6-methyladenine: the other methylated base of
88 DNA. *BioEssays* **28,** 309–315 (2006).
89 40. Ratel, D. *et al.* Undetectable levels of N6-methyl adenine in mouse DNA: Cloning and analysis of
90 PRED28, a gene coding for a putative mammalian DNA adenine methyltransferase. *FEBS Letters*
91 **580,** 3179–3184 (2006).
92 41. Low, D. A., Weyand, N. J. & Mahan, M. J. Roles of DNA adenine methylation in regulating bacterial
93 gene expression and virulence. *Infect. Immun.* **69,** 7197–7204 (2001).
94 42. Li, X., Baker-Andresen, D., Zhao, Q., Marshall, V. & Bredy, T. W. Methyl CpG Binding Domain
95 Ultra-Sequencing: a novel method for identifying inter-individual and cell-type-specific variation in
96 DNA methylation. *Genes, Brain and Behavior* **13,** 721–731 (2014).
97 43. Cheng, X. & Roberts, R. J. AdoMet-dependent methylation, DNA methyltransferases and base
98 flipping. *Nucl. Acids Res.* **29,** 3784–3795 (2001).
99 44. Diekmann, S. DNA methylation can enhance or induce DNA curvature. *EMBO J.* **6,** 4213–4217
00 (1987).
01 45. Coulombe, B. & Burton, Z. F. DNA bending and wrapping around RNA polymerase: a 'revolutionary'
02 model describing transcriptional mechanisms. *Microbiol. Mol. Biol. Rev.* **63,** 457–478 (1999).
03 46. Guo, Q., Lu, M. & Kallenbach, N. R. Effect of hemimethylation and methylation of adenine on the
04 structure and stability of model DNA duplexes. *Biochemistry* (1995).
05 47. Workman, J. L. Nucleosome displacement in transcription. *Genes Dev.* **20,** 2009–2017 (2006).
06 48. Navarro, P. & Avner, P. An embryonic story: analysis of the gene regulative network controlling Xist
07 expression in mouse embryonic stem cells. *BioEssays* **32,** 581–588 (2010).
08 49. Funahashi, N. *et al.* YY1 positively regulates human UBIAD1 expression. *Biochem. Biophys. Res.*
09 *Commun.* **460,** 238–244 (2015).
10 50. Makhlouf, M. *et al.* A prominent and conserved role for YY1 in Xist transcriptional activation. *Nat*
11 *Comms* **5,** 4878 (2014).
12 51. Chapman, A. G., Cotton, A. M., Kelsey, A. D. & Brown, C. J. Differentially methylated CpG island
13 within human XIST mediates alternative P2 transcription and YY1 binding. *BMC Genetics 2014 15:1*
14 **15,** 89 (2014).
15 52. Kellinger, M. W. *et al.* 5-formylcytosine and 5-carboxylcytosine reduce the rate and substrate
16 specificity of RNA polymerase II transcription. *Nat. Struct. Mol. Biol.* **19,** 831–833 (2012).

17 53. Wang, L. *et al.* Molecular basis for 5-carboxycytosine recognition by RNA polymerase II elongation
18 complex. *Nature* **523,** 621–625 (2015).
19 54. Tian, F. *et al.* Dynamic chromatin remodeling events in hippocampal neurons are associated with
20 NMDA receptor-mediated activation of Bdnf gene promoter 1. *J. of Neurochem.* **109,** 1375–1388
21 (2009).
22

23







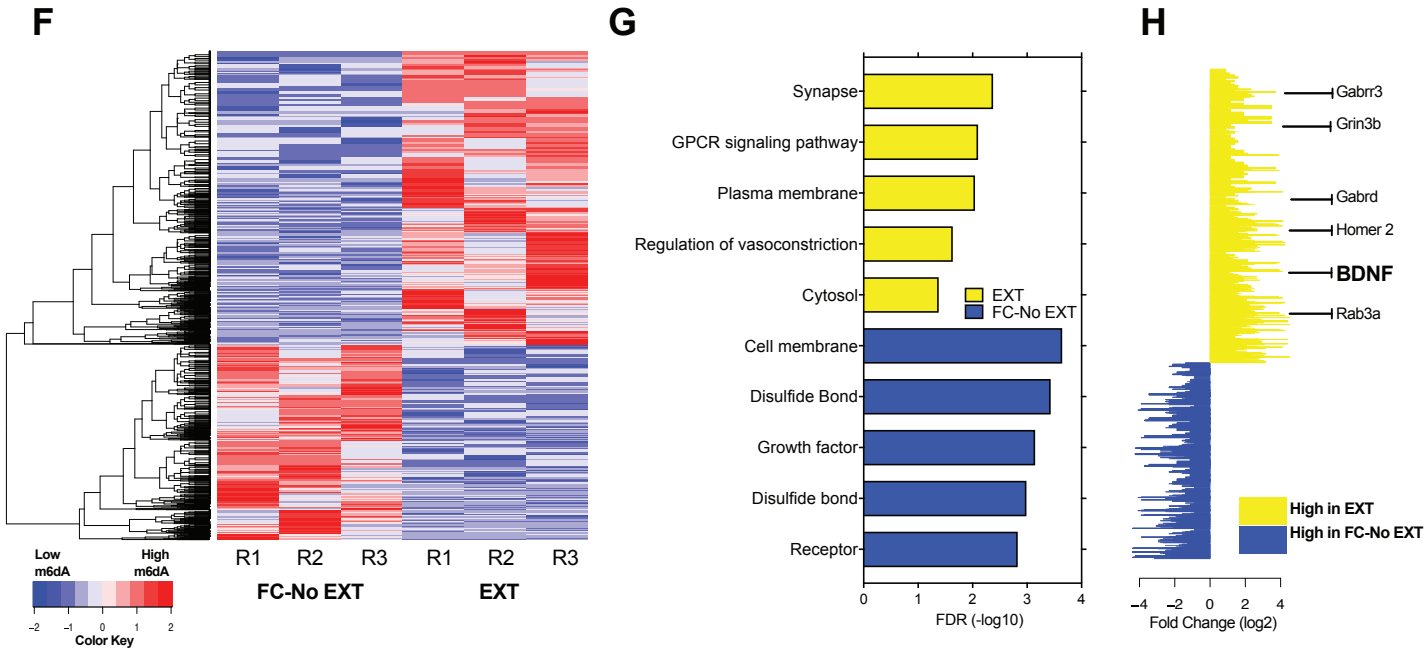


Fig 1

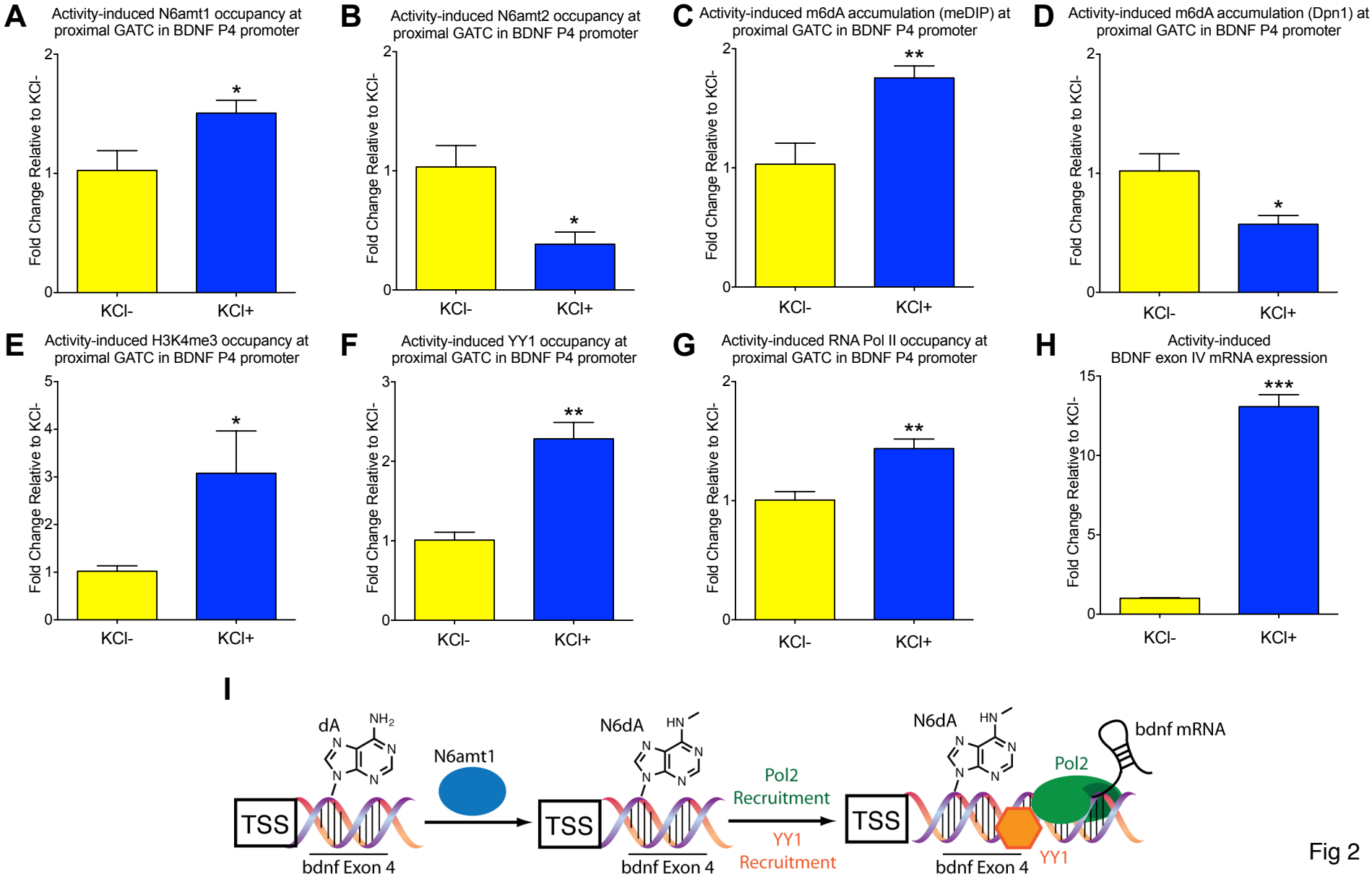

A
Activity-induced N6amt1 occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to KCl-
KCl-
KCl+
B
Activity-induced N6amt2 occupancy at proximal GATC in BDNF P4 promoter
C
Activity-induced m6dA accumulation (meDIP) at proximal GATC in BDNF P4 promoter
D
Activity-induced m6dA accumulation (Dpn1) at proximal GATC in BDNF P4 promoter
E
Activity-induced H3K4me3 occupancy at proximal GATC in BDNF P4 promoter
F
Activity-induced YY1 occupancy at proximal GATC in BDNF P4 promoter
G
Activity-induced RNA Pol II occupancy at proximal GATC in BDNF P4 promoter
H
Activity-induced BDNF exon IV mRNA expression
I
dA
N6amt1
N6dA
Pol2 Recruitment
YY1 Recruitment
bdnf mRNA
Pol2
YY1
TSS
bdnf Exon 4

Fig 2

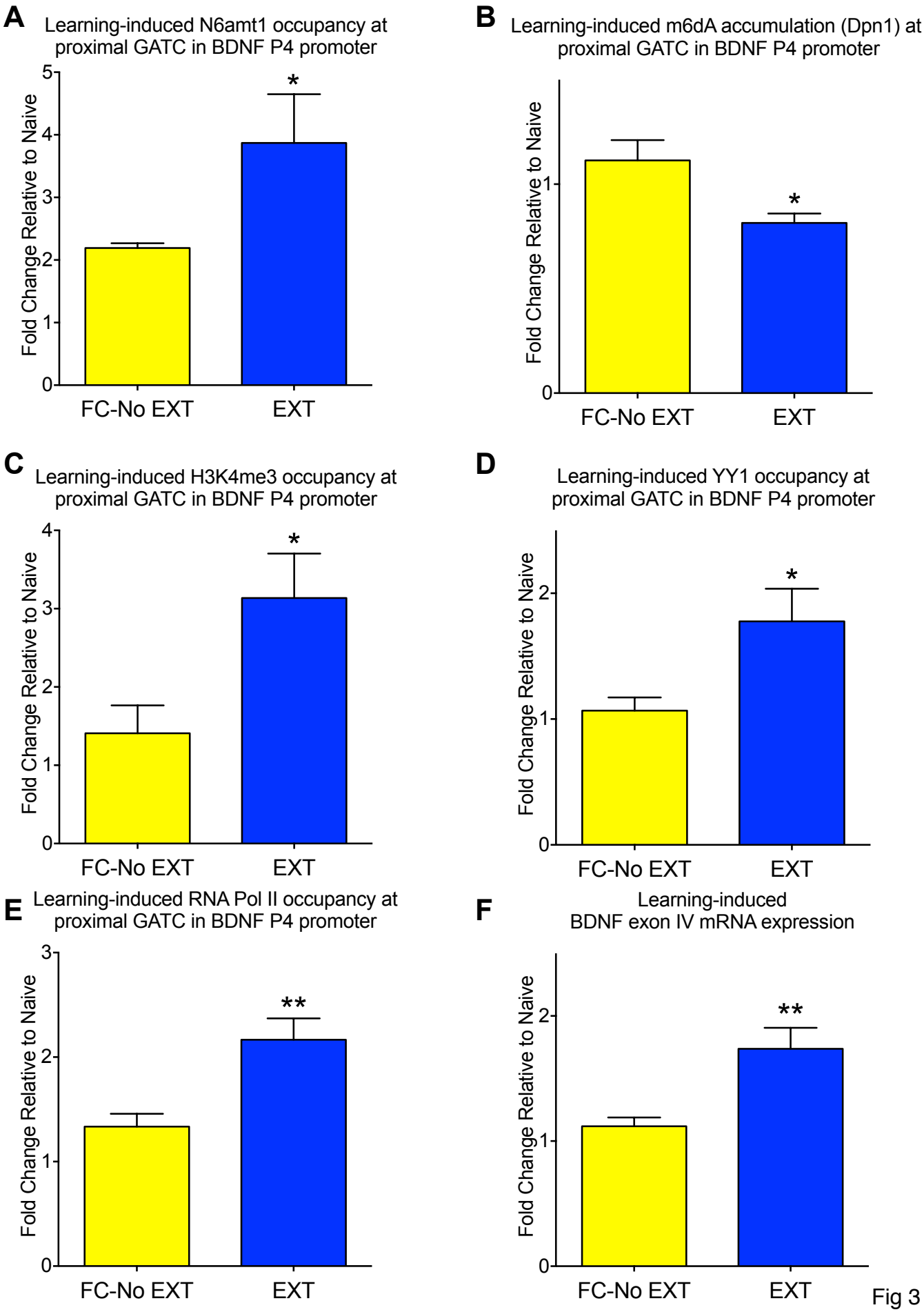
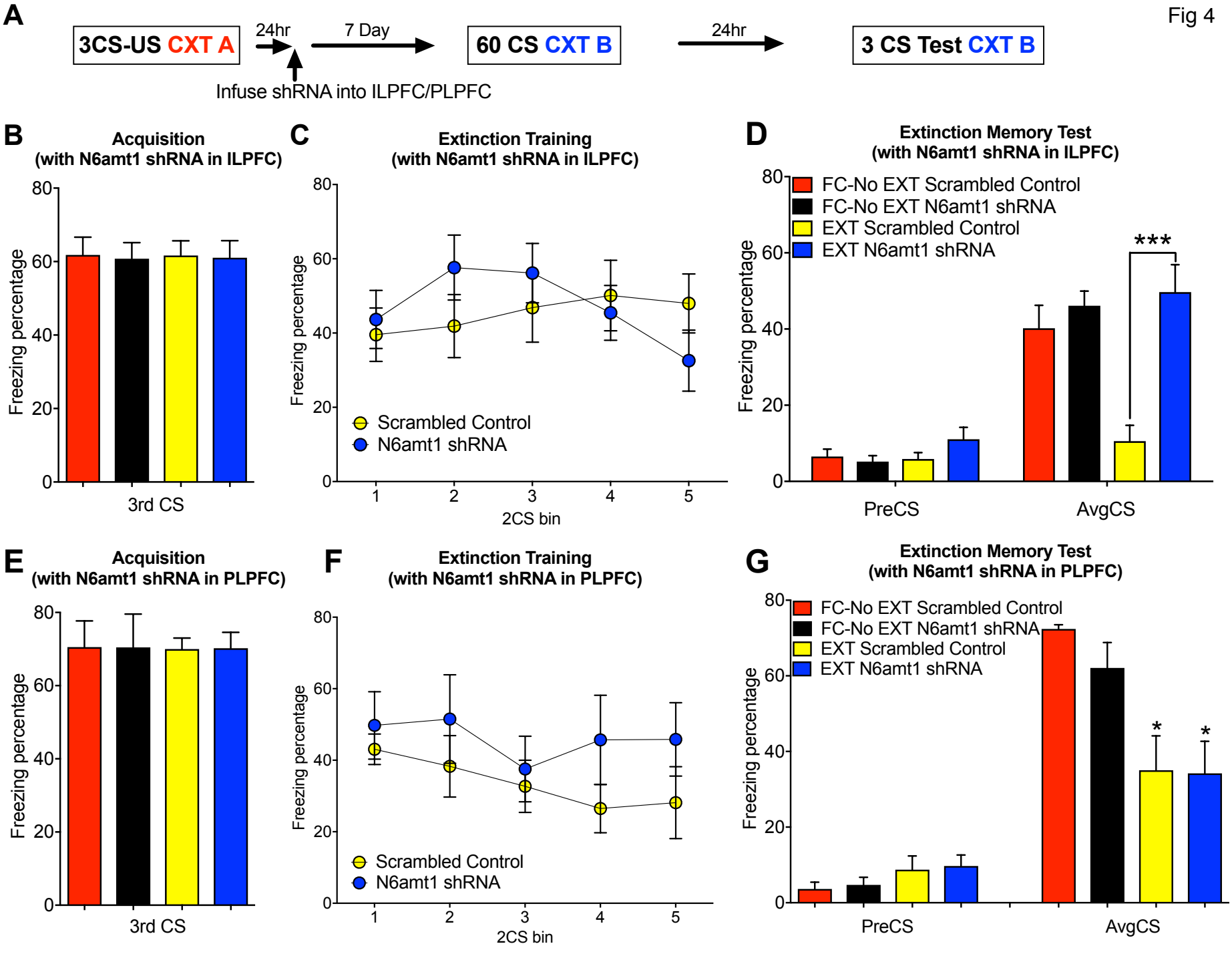

A
Learning-induced N6amt1 occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
2
3
4
5
*
FC-No EXT
EXT
B
Learning-induced m6dA accumulation (Dpn1) at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
*
FC-No EXT
EXT
C
Learning-induced H3K4me3 occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
2
3
4
*
FC-No EXT
EXT
D
Learning-induced YY1 occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
2
*
FC-No EXT
EXT
E
Learning-induced RNA Pol II occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
2
3
**
FC-No EXT
EXT
F
Learning-induced BDNF exon IV mRNA expression
Fold Change Relative to Naive
0
1
2
**
FC-No EXT
EXT

Fig 3


A
3CS-US CXT A
24hr
7 Day
60 CS CXT B
24hr
3 CS Test CXT B
Infuse shRNA into ILPFC/PLPFC
B
Acquisition
(with N6amt1 shRNA in ILPFC)
Freezing percentage
3rd CS
C
Extinction Training
(with N6amt1 shRNA in ILPFC)
Freezing percentage
Scrambled Control
N6amt1 shRNA
2CS bin
D
Extinction Memory Test
(with N6amt1 shRNA in ILPFC)
FC-No EXT Scrambled Control
FC-No EXT N6amt1 shRNA
EXT Scrambled Control
EXT N6amt1 shRNA
***
Freezing percentage
PreCS
AvgCS
E
Acquisition
(with N6amt1 shRNA in PLPFC)
Freezing percentage
3rd CS
F
Extinction Training
(with N6amt1 shRNA in PLPFC)
Freezing percentage
Scrambled Control
N6amt1 shRNA
2CS bin
G
Extinction Memory Test
(with N6amt1 shRNA in PLPFC)
FC-No EXT Scrambled Control
FC-No EXT N6amt1 shRNA
EXT Scrambled Control
EXT N6amt1 shRNA
*
*
Freezing percentage
PreCS
AvgCS

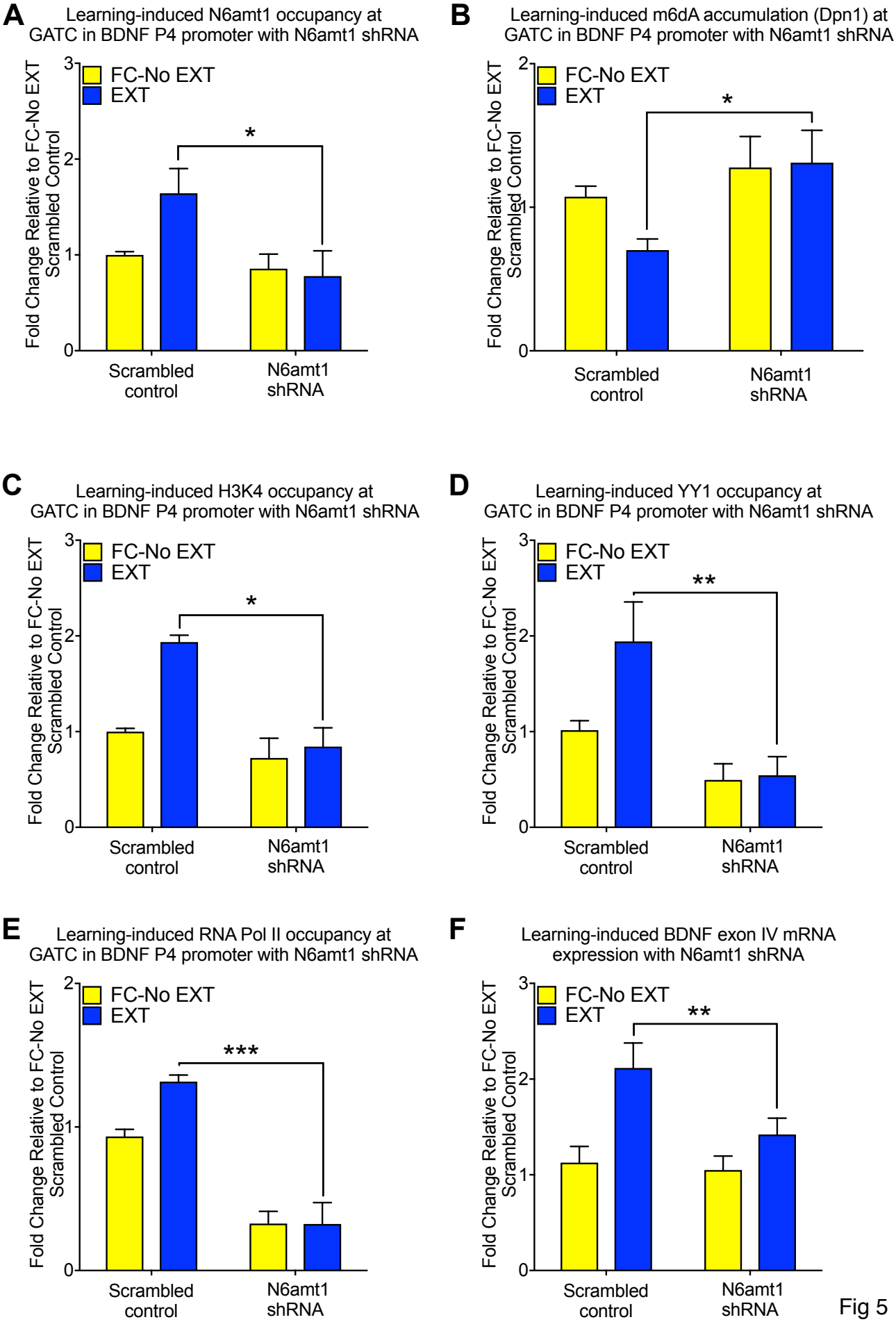

A
Learning-induced N6amt1 occupancy at GATC in BDNF P4 promoter with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
*
Scrambled control
N6amt1 shRNA
B
Learning-induced m6dA accumulation (Dpn1) at GATC in BDNF P4 promoter with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
*
Scrambled control
N6amt1 shRNA
C
Learning-induced H3K4 occupancy at GATC in BDNF P4 promoter with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
*
Scrambled control
N6amt1 shRNA
D
Learning-induced YY1 occupancy at GATC in BDNF P4 promoter with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
**
Scrambled control
N6amt1 shRNA
E
Learning-induced RNA Pol II occupancy at GATC in BDNF P4 promoter with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
***
Scrambled control
N6amt1 shRNA
F
Learning-induced BDNF exon IV mRNA expression with N6amt1 shRNA
FC-No EXT
EXT
Fold Change Relative to FC-No EXT Scrambled Control
**
Scrambled control
N6amt1 shRNA

Fig 5

Fig. 1. Experience-dependent redistribution of m6dA deposition within prefrontal cortical neurons that have been activated by extinction learning. (A) Using flow cytometry coupled with DpnI-seq, we generated a fear extinction learning-induced genome-wide profile of m6dA deposition in a cell-type-specific manner and at single-base resolution. (B) The nucleotide composition of all potential m6dA sites is biased toward the GATC motif. (C) m6dA deposition is primarily located in the promoter and CDS regions with a significant drop at the TSS and TES. (D) Frequency plots demonstrating that m6dA is enriched at +1bp from TSS, and (E) exhibits a significant increase in deposition at +4bp from the start codon. (F) Representative heat map of genome-wide m6dA enrichment within active neurons after behavioral training. (G) Gene ontology analysis of 10 gene clusters associated with m6dA deposition in EXT vs. FC-No EXT groups. (H) Representative list of genes that exhibit a significant increase in the accumulation of m6dA and that have been associated with synaptic function, learning and memory.

Fig. 2. Activity-induced N6amt1 occupancy and the accumulation of m6dA are associated with increased bdnf exon IV mRNA expression. KCl-induced depolarization leads to (A) an increase in N6amt1 (*p<.05), but (B) decrease on N6amt2 (*p<.05) occupancy, (C) increased deposition of m6dA (**p<.01), (D) an increase in m6dA at a GATC site adjacent to the consensus sequence for YY1 (*p<.05), (E) increased occupancy of the chromatin mark H3K4^{me3} (*p<.05), (F) increased recruitment of YY1 proximal to the m6dA modified adenine (**p<.01), (G) a concomitant increase in the presence of Pol II (**p<.01), (H) a correlated increase in the induction of bdnf exon IV mRNA expression (***p<.001). (I) A schematic of activity-dependent deposition of m6dA by N6amt1, which occurs in a tightly regulated, and spatiotemporally controlled manner

through locus-specific chromatin modification and the recruitment of activating transcription machinery. (All n=3-4/group, Error bars represent SEM)

Fig. 3. Extinction learning-induced accumulation of m6dA is associated with bdnf exon IV mRNA expression. Fear extinction learning (EXT), relative to mice fear conditioned and exposed to a novel context (FC-No EXT), led to (A) an selective increase in N6amt1 occupancy (*p<.05), (B) increased m6dA at the previously identified GATC site (*p<.05), (C) a significant increase in $H3K4^{me3}$ occupancy (*p<.05), (D) an increase in the recruitment of YY1 (*p<.05), (E) an increase in Pol II occupancy (**p<.01). (F) a significant increase in bdnf exon IV mRNA expression within the ILPFC (**p<.01). (All n=6-8/group, Error bars represent SEM)

Fig. 4. N6amt1-mediated accumulation of m6dA is required for fear extinction memory and for learning-induced bdnf exon IV mRNA expression in the ILPFC. (A) Schematic of the behavioral protocol used to test the effect of lentiviral-mediated knockdown of N6amt1 in the ILPFC or PLPFC on fear extinction memory. (B-C) There was no effect of N6amt1 shRNA on within-session performance during the first 10 conditioned stimulus exposures during fear extinction training. (D) Although there was no effect of N6amt1 shRNA on fear expression in mice that had been fear conditioned and exposed to a novel context without extinction training, N6amt1 knockdown led to a significant impairment in fear extinction memory (*p<.05). (E-G) N6amt1 knockdown in the PLPFC had no effect on the formation of fear extinction memory (*p<.05). (All n=8/group, Error bars represent SEM).

Fig. 5. N6amt1 knockdown prevents the learning-induced accumulation of m6dA and related changes in chromatin and transcriptional landscape associated with

the BDNF P4 promoter. Following ILPFC infection with N6amt1 shRNA, N6amt1 shRNA blocked (A) increase of N6amt1 occupancy (two-way ANOVA $F_{1,12}$ = 6.360, p<.05; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, **p<.01) and (B) the deposition of m6dA (two-way ANOVA $F_{1,12}$ = 6.063, p<.05; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, *p<.05), or accumulation of $H3K4me^3$ (two-way ANOVA $F_{1,12}$ = 21.31, p<.0005; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, ***p<.001), YY1 (two-way ANOVA $F_{1,12}$ = 14.87, p<.005; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, **p<.005) and RNA Pol II (two-way ANOVA $F_{1,12}$ = 75.13, p<.0001; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, ****p<.0001) occupancy at the proximal GATC site within the bdnf P4 promoter (All n=4/group, Error bars represent SEM). Also, N6amt1 shRNA inhibited the bdnf exon 4 expression (two-way ANOVA $F_{1,12}$ = 4.062, p<.05; Fisher's posthoc test; Scrambled control EXT vs. N6amt1 shRNA EXT, *p<.05)