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

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We have discovered that the recently identified mammalian DNA modification N6-methyl-2'-deoxyadenosine (m6dA) drives activity-induced gene expression in the adult brain and is associated with the formation of fear extinction memory in C57/Bl6 mice. In activated primary cortical neurons, m6dA accumulates within the P4 promoter of the gene encoding brain-derived neurotrophic factor (bdnf), which is associated with an active chromatin state, as well as the recruitment of the activating transcription factor Yin-Yang 1 and RNA polymerase II, thereby promoting bdnf exon IV mRNA expression. Lentiviral-mediated knockdown of a potential adenine methyltransferase, N6amt1, blocks the effect of neuronal activation on m6dA and its related chromatin and transcriptional machinery *in vitro*. These effects are recapitulated in the adult brain, where the extinction learning-induced N6amt1-mediated accumulation of m6dA in the infralimbic prefrontal cortex also enhances the expression of bdnf exon IV and is necessary for the formation of fear extinction memory.

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 (*1-3)*. Although it is known that there are more than 20 DNA modifications (4), 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 (*5-7*). 5mC and 5hmC are highly prevalent in neurons relative to other cell types (*5*,*8*) and both modifications are regulated in response to learning (*9-11*). Despite this, a complete understanding of how DNA methylation controls neuronal gene expression to facilitate memory formation is severely lacking. Beyond cytosine, recent findings indicate that N6-methyl-2'-deoxyadenosine (m6dA) is also regulated in eukaryotic DNA. In *C. reindardtii,* m6dA accumulates at transcription start sites (*12*) and in *Drosophila*, m6dA increases across development and is enriched within transposable elements (*13*). Furthermore, m6dA appears to be involved in reproductive viability in *C. elegans* (*14*). These observations have led to the speculation that m6dA may be a critical regulator of gene expression in lower eukaryotes. Although m6dA is present in the genome of vertebrates (*15*), whether it is dynamic in the genome of higher eukaryotes and associated with behavioral adaptation has yet to be determined.

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 activating transcription in lower eukaryotes (12), we hypothesized that m6dA may also be fundamental for governing experience-dependent gene expression within the mammalian genome. We therefore set out to explore the role of m6dA within neurons and to elucidate whether it is involved in the regulation of activity-induced gene expression (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 in GATC linker sequences (Fig. 1B) (*12*), which revealed evidence in favor of m6dA as a modified base in neuronal DNA (Fig. 1C). Mass spectrometry was then used to quantify m6dA (Fig. 1D). The presence of m6dA was verified and estimated to occur within ~46 per 10^6 dNs (~280,000 m6dA)

across the neuronal genome. We next performed a dot blot assay in order to determine whether m6dA is dynamic in neuronal DNA. This revealed a significant activity-induced global accumulation of m6dA, which was evident in primary cortical neurons in culture in response to neural activation (7DIV, 20mM KCl, 7 hrs, Fig 1E, t-test, t_4=2.54, p<.05). Together, these data strongly suggest that m6dA is both a dynamic and prevalent base modification. The relative abundance and distribution of m6dA in the mammalian genome may therefore be cell-type specific, with greater accumulation in post-mitotic neurons compared to other cell types.

In order to elucidate the underlying mechanism by which m6dA accumulates in the mammalian genome, we examined the expression of two potential mammalian adenine-specific methyltransferases in primary cortical neurons. 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 (*17*); however, whether the same process occurs in mammalian DNA remains equivocal (*18*). 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 (*16*). N6amt2, which shares a highly conserved methyltransferase domain, is also expressed in the mouse brain (http://mouse.brain-map.org/experiment/show?id=69837159). Based on these observations, N6amt1 and N6amt2 were selected for an analysis of their role in dynamically regulating the accumulation of m6dA in the mammalian brain.

N6amt1 exhibited a time-dependent increase in mRNA levels in primary cortical neurons in response to KCl-induced depolarization (Suppl. Fig 1A, t-test, t_6=4.14, p<.01). There was no effect on N6amt2 (Suppl. Fig. 1B). Behavioral training led to a significant increase in N6amt1 mRNA expression in the infralimbic prefrontal cortex (ILPFC), *in vivo* (Suppl. Fig. 1C, t-test, t_6=3.64, p<.01), again with no detectable change in N6amt2 (Suppl. Fig. 1D). In contrast to the ILPFC, hippocampal N6amt1 and N6amt2 mRNA showed no change in expression in response to behavioral training (Suppl. Fig. 1E-F). These findings indicate that the gene encoding N6amt1, but not N6amt2, is inducible in primary cortical neurons and is expressed in the infralimbic prefrontal cortex (ILPFC) in response to extinction learning. The data also suggest that N6amt1 might serve to

regulate the accumulation of m6dA in the neuronal genome in a dynamic, context- and experience-dependent manner. To test this possibility, we overexpressed N6amt1 (N6amt1ox) within HEK cells (Suppl. Fig. S2A-C). As evidenced by dot blot assay, N6amt1ox led to a significant increase in the global level of m6dA (Suppl. Fig. S2D). To further investigate the functional relevance of N6amt1, we co-transfected primary cortical neurons with N6amt1ox and N6amt1 shRNA, which blocked the N6am1ox-mediated increase in m6dA (Suppl. Fig. S2E). Taken together, these data demonstrate a role for N6amt1 in regulating the activity-induced deposition of m6dA within post-mitotic cortical neurons.

Bdnf is the most widely expressed inducible neurotrophin in the central nervous system (*19*), and is directly involved in learning and memory (*20*). In the adult brain, 5mC within BDNF gene promoters is altered by experience (*21*), and this regulation appears to be necessary for the regulation of gene expression underlying remote memory (*9*). The BDNF locus comprises at least eight homologous noncoding exons that contribute to alternate 5'UTRs, and a ninth that contributes a protein coding sequence and a 3'UTR (*22*, *23*). The complex structure of this locus has led to the idea that bdnf expression may be driven by unique DNA modifications that guide distinct sets of transcription factor complexes to initiate the transcription of the various isoforms (*24*), 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*, *26*). The bdnf exon IV locus therefore represents a prototypical epigenetically responsive gene, ideally suited to examine the functional relevance of dynamic changes in m6dA in DNA that are induced by experience.

We therefore asked whether an N6amt1-mediated accumulation of m6dA at the BDNF exon IV locus occurs in response to neuronal activation. Chromatin immunoprecipitation analysis revealed an increase in N6amt1 occupancy (Fig. 2A, t-test, t_6=2.77, p<.05), but not N6amt2 (Fig. 2B), at a region of the BDNF P4 promoter immediately downstream of the transcription start site (TSS). 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 used. KCl-induced depolarization led to an increase in m6dA at a GATC site adjacent to the consensus sequence for

the activating transcription factor Yin-Yang (YY1) (Fig 2D. t-test, t_4=2.76, p<.05). 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 adenine (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, which is strongly predictive of transcriptional elongation (Fig. 2G, t-test, t_4=4.04, p<.01). 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, the observed m6dA deposition and associated changes did not occur at a distal GATC site located within the BDNF P4 promoter 1000bp upstream of the TSS (Suppl. Fig. S3A-E).

In order to gain deeper insight into the mechanism by which dynamic changes in m6dA influence bdnf exon IV expression, an N6amt1 shRNA was generated according to our previously published protocols and its efficiency was validated *in vitro* (Suppl. Fig. S4). In primary cortical neurons infected with N6amt1 shRNA, the observed activity-dependent N6amt1 occupancy and m6dA deposition at the exon IV locus were eliminated (Suppl. Fig S5A-B) and 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 S5C-E). The change in the local chromatin landscape and recruitment of transcription factors led to reduced bdnf exon IV mRNA expression (Suppl. Fig S5F). However, overexpression of N6amt1 in primary cortical neurons *in vitro* did not increase the deposition of m6dA at the same GATC site *in vitro*, even though the accumulation of m6dA was blocked in the presence of N6amt1 shRNA (Suppl. Fig. S2F). Together, these data demonstrate that one critically important role for an N6amt1-associated accumulation of m6dA in DNA is to promote bdnf expression in response to neural activation. Furthermore, our findings suggest that the activity-dependent deposition of m6dA by N6amt1 likely occurs in a tightly regulated and spatiotemporally controlled manner through locus-specific chromatin modification and the recruitment of activating transcription machinery (Fig 2I).

It is important to note that although we provide strong evidence to suggest that N6amt1 is necessary for the dynamic accumulation of m6dA, at this stage we cannot conclude whether this occurs via its putative

adenine methyltransferase domain or by some other protein-DNA interaction. Indeed, the latter is a distinct possibility given that other epigenetic regulatory proteins known to control DNA modification, such as the Tet family of dioxygenases, can exert their influence independent of their catalytic activity (*27,28*). By using recombinant protein and *in vitro* methyltransferase assays, future studies will aim to determine whether N6amt1 coordinates m6dA deposition either as part of a larger complex or directly through enzymatic activity.

To determine whether the observed effects in primary cortical neurons are also functionally relevant in the adult brain, we next examined the role of m6dA in regulating extinction learning-induced bdnf exon IV mRNA expression within the ILPFC. 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, which depend on 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 (*29*). A variety of epigenetic mechanisms in the ILPFC have been implicated in fear extinction (*11*, *25*, *30*). This behavioral model therefore serves as a robust platform to interrogate the role of epigenetic mechanisms in a critically important memory process of critical importance.

Similar to the effect of KCl-induced depolarization on m6dA and correlated gene expression *in vitro*, fear extinction learning (EXT) led to an increase in N6amt1 occupancy relative to that in mice which had been fear conditioned and only exposed to a novel context (FC-No EXT) (Fig. 3A, t-test, t_{10}=1.87, p<.05), This was accompanied by increased deposition of m6dA (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 a 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).

Having established a relationship between an 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 affected the formation of fear extinction memory (Fig. 4A). We first validated the efficacy of the knockdown construct, 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. 6A-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, preCS). However, in mice that had been extinction trained in the presence of N6amt1 shRNA, there was a highly significant impairment in fear extinction memory (Fig. 4D-right, two-way ANOVA $F_{1,28}$= 9.18, p<.01; Bonferroni's posthoc test; EXT scrambled vs. EXT shRNA, p<.001). As a control, we also generated an N6amt2 shRNA and observed no effect of N6amt2 knockdown in the ILPFC on the formation of fear extinction memory (Fig. 4E-G). Infusion of the N6amt1 shRNA into the prelimbic region of the prefrontal cortex, a brain region immediately dorsal to the ILPFC and not required for the acquisition or expression of fear extinction memory, also had no effect (Suppl. 7D). These data demonstrate an important role for the N6amt1-mediated accumulation of m6dA in the ILPFC in regulating the formation of fear extinction memory. At this stage, however, a more generalized role for m6dA in other forms of learning and memory cannot be ruled out.

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, p<.01) and the accumulation of m6dA (Fig. 5B, p<.05). N6amt1 knockdown also blocked the extinction-learning induced increase $H3K4^{me3}$ (Fig. 5C, **p<.01), YY1 (Fig. 5D, p<.01), and Pol II occupancy at the BDNF P4 promoter (Fig. 5E, p<.001). Finally, similar to the *in vitro* findings, N6amt1 knockdown blocked the effect of extinction training on bdnf exon IV mRNA expression (Fig. 5F, p<.01). Taken together, these findings suggest that a dynamic, learning-induced, accumulation of m6dA in the adult ILPFC, mediated in part by N6amt1, plays a critical role in regulating experience-dependent bdnf exon IV expression and the formation of extinction memory.

YY1 initiates context-specific gene expression through interaction with Pol II (*31-33*), which is dependent on DNA methylation and changes in the local chromatin landscape (*34-36*). In agreement with these findings, the activity-induced expression of bdnf exon IV 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. S4A-B and Fig. 4H-I). This is also associated with the presence of $H3K4^{me3}$, an epigenetic mark that reflects an active chromatin state (Suppl. Fig. S4C and Fig. 4J), and is accompanied by increased occupancy of the transcription factor YY1 and Pol II at the same locus (Suppl. Fig. S4C-D and Fig 4K-l). 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 (*12*).

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 (*13*, *37*, *38*). 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 a 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 (*39*). 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 (*2*).

In summary, the discovery of activity-induced accumulation of m6dA in the mammalian genome that is required for memory formation dramatically expands the scope of experience-dependent DNA modifications in the brain. It is evident that the information-processing capacity of DNA is far more complex than current

perspectives, and our finding that m6dA is a potent regulator of gene expression likely represents the tip of the iceberg for this newly discovered epigenetic mark. We predict that a large number of dynamic and functional modifications on *all four* canonical nucleobases remain to be discovered, and it will be within the realm of cognition and memory where the impact of these novel epigenetic purveyors of genomic and behavioral diversity will be most significant.

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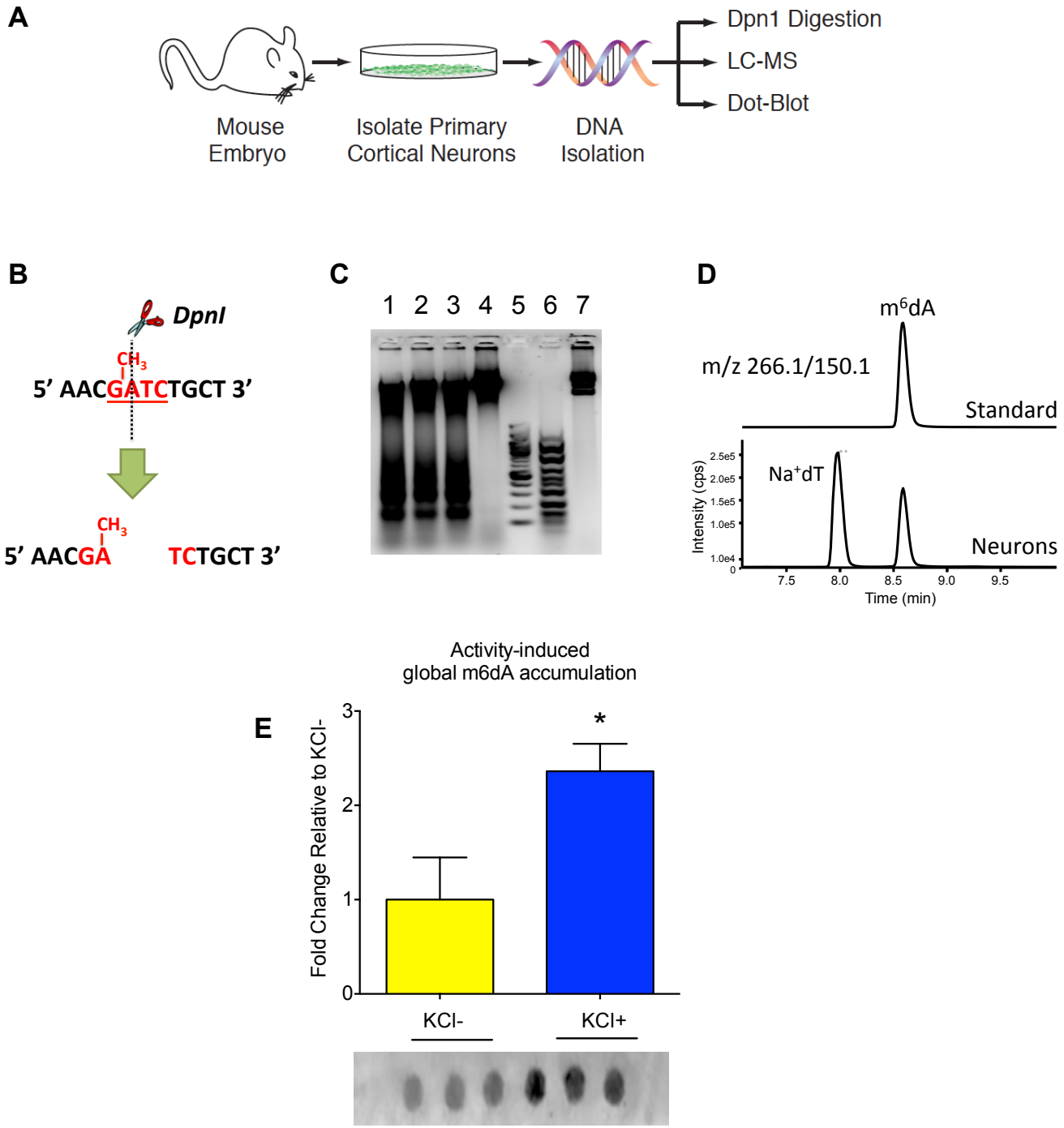
Fig. 1


A
Mouse Embryo
Isolate Primary Cortical Neurons
DNA Isolation
Dpn1 Digestion
LC-MS
Dot-Blot
B
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CH3
5' AACGATCTGCT 3'
CH3
5' AACGA
TCTGCT 3'
C
1 2 3 4 5 6 7
D
m6dA
m/z 266.1/150.1
Standard
Na+dT
Neurons
Intensity (cps)
2.5e5
2.0e5
1.5e5
1.0e5
1.0e4
0
7.5
8.0
8.5
9.0
9.5
Time (min)
Activity-induced global m6dA accumulation
E
Fold Change Relative to KCl-
3
2
1
0
*
KCl-
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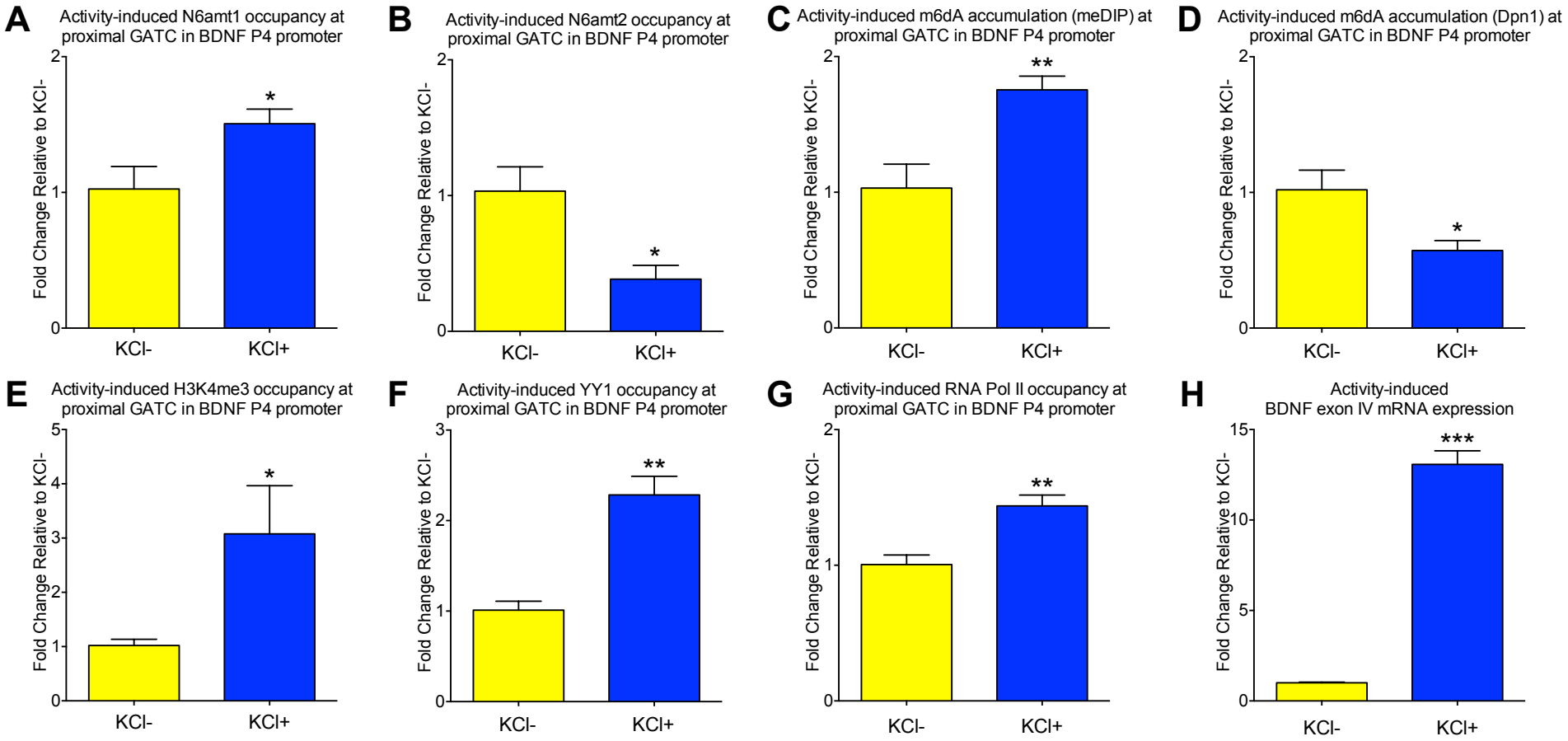
Fig. 2


A
Activity-induced N6amt1 occupancy at proximal GATC in BDNF P4 promoter
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
Fold Change Relative to KCl-
KCl-
KCl+
*
**
***

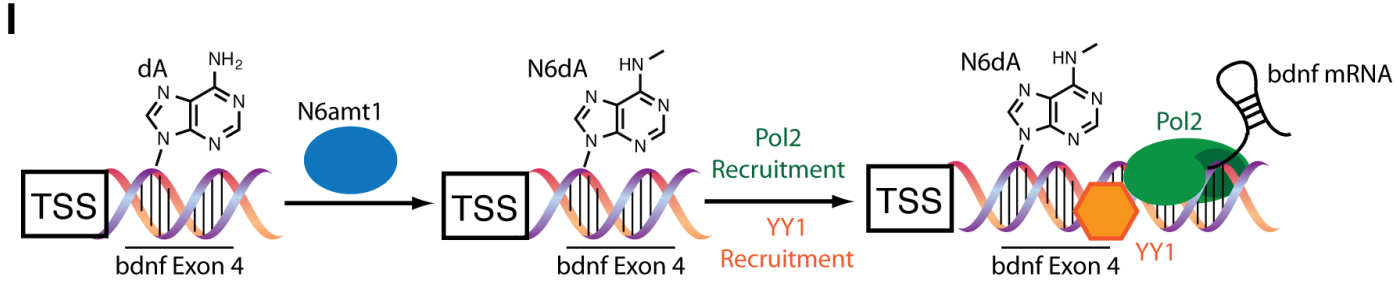

I
dA
NH2
N6amt1
N6dA
HN
Pol2
Recruitment
YY1
Recruitment
bdnf mRNA
Pol2
YY1
TSS
bdnf Exon 4

Fig. 3

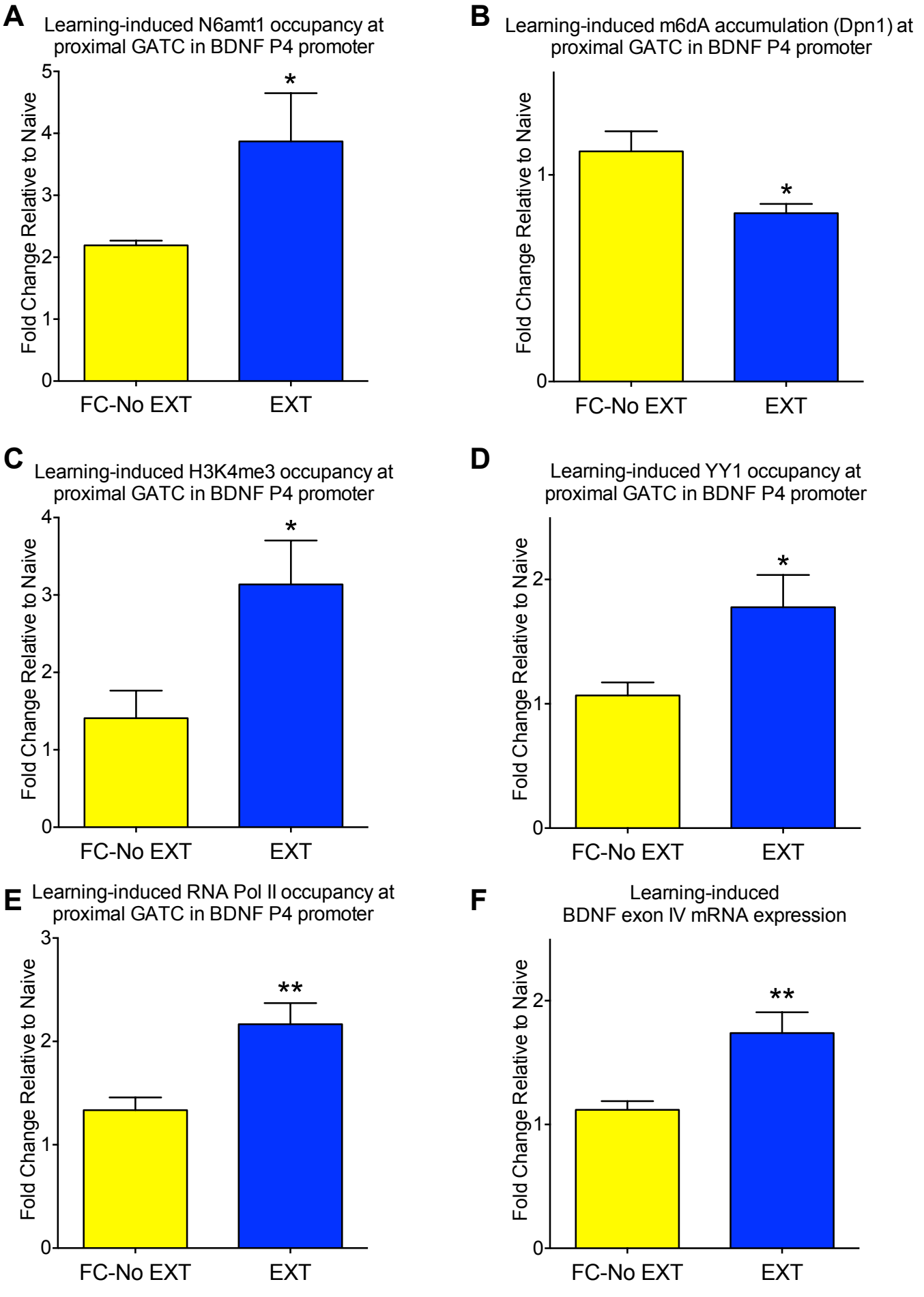
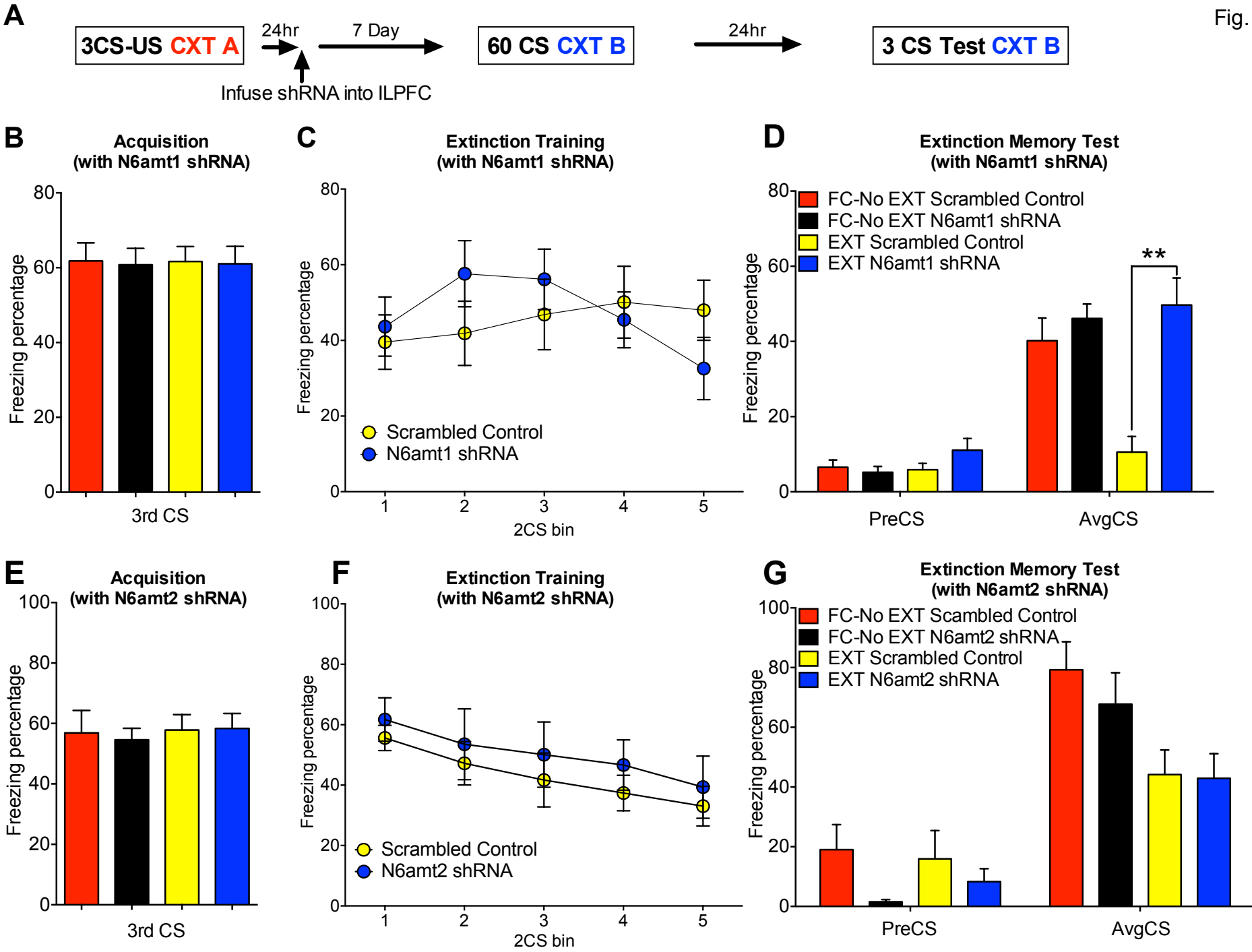
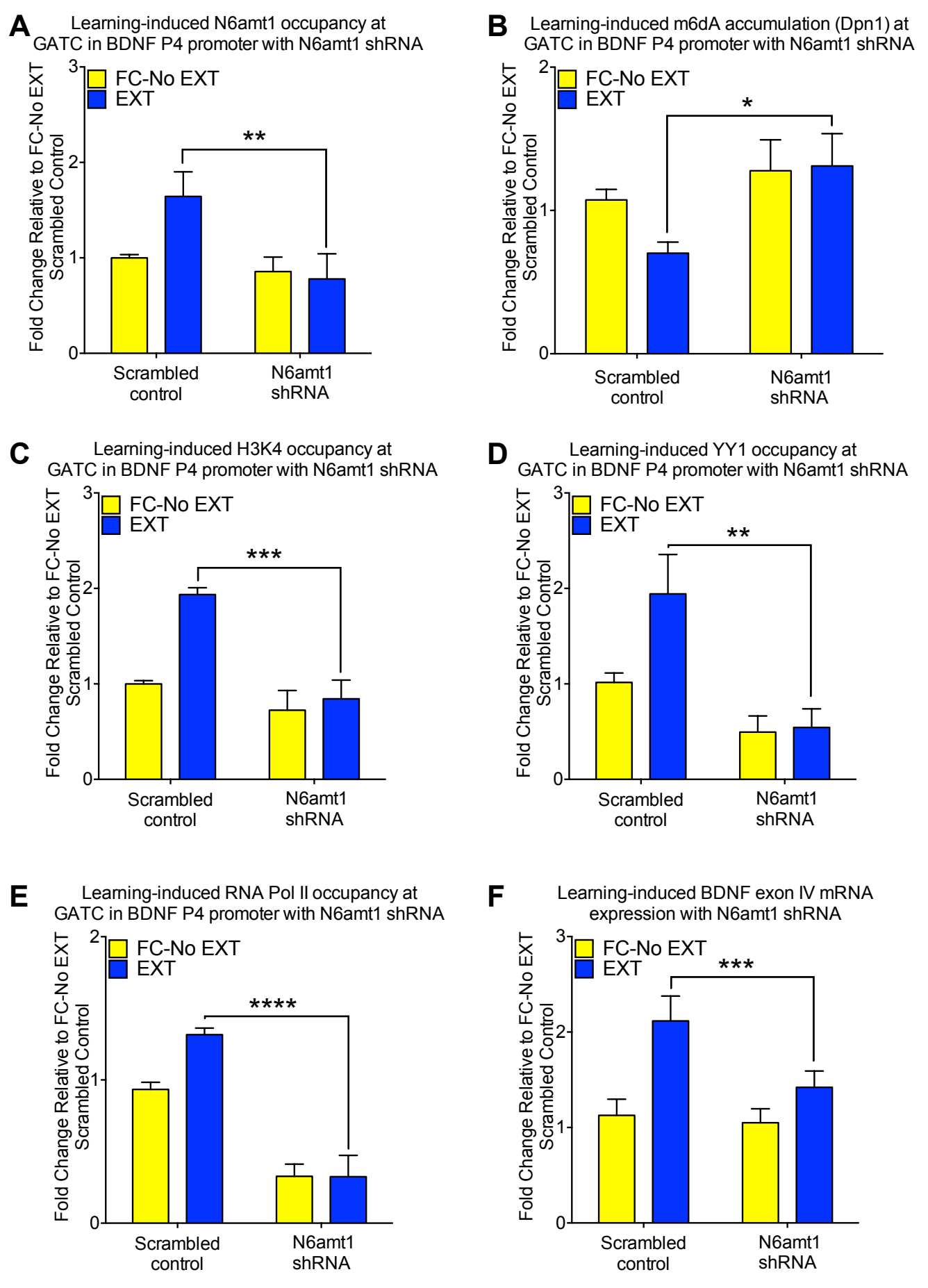

A
Learning-induced N6amt1 occupancy at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
2
3
4
5
*
FC-No EXT
EXT
B
Learning-induced m6dA accumulation (Dpn1) at proximal GATC in BDNF P4 promoter
Fold Change Relative to Naive
0
1
*
FC-No EXT
EXT
C
Learning-induced H3K4me3 occupancy at 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. 4


A
3CS-US CXT A
24hr
7 Day
60 CS CXT B
24hr
3 CS Test CXT B
Infuse shRNA into ILPFC
B
Acquisition
(with N6amt1 shRNA)
Freezing percentage
3rd CS
C
Extinction Training
(with N6amt1 shRNA)
Freezing percentage
Scrambled Control
N6amt1 shRNA
2CS bin
D
Extinction Memory Test
(with N6amt1 shRNA)
FC-No EXT Scrambled Control
FC-No EXT N6amt1 shRNA
EXT Scrambled Control
EXT N6amt1 shRNA
**
Freezing percentage
PreCS
AvgCS
E
Acquisition
(with N6amt2 shRNA)
Freezing percentage
3rd CS
F
Extinction Training
(with N6amt2 shRNA)
Freezing percentage
Scrambled Control
N6amt2 shRNA
2CS bin
G
Extinction Memory Test
(with N6amt2 shRNA)
FC-No EXT Scambled Control
FC-No EXT N6amt2 shRNA
EXT Scrambled Control
EXT N6amt2 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
0
1
2
3
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
0
1
2
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
0
1
2
3
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
0
1
2
3
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
0
1
2
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
0
1
2
3
Scrambled control
N6amt1 shRNA