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M6A RNA Methylation-Based Epitranscriptomic Modifications in Plasticity-Related Genes via miR-124-C/EBPα-FTO-Transcriptional Axis in the Hippocampus of Learned Helplessness Rats

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Abstract

Background: Impaired synaptic plasticity has been linked to dynamic gene regulatory network changes. Recently, gene regulation has been introduced with the emerging concept of unique N6-methyladenosine (m^6A)-based reversible transcript methylation. In this study, we tested whether m^6A RNA methylation may potentially serve as a link between the stressful insults and altered expression of plasticity-related genes.
Methods: Expression of plasticity genes Nr3c1, Creb1, Ntrk2; m6A-modifying enzymes Fto, methyltransferase like (Mettl)-3 and 14; DNA methylation enzymes Dnmt1, Dnmt3a; transcription factor C/ebp-α; and miRNA-124-3p were determined by quantitative polymerase chain reaction (qPCR) in the hippocampus of rats that showed susceptibility to develop stress-induced depression (learned helplessness). M^6A methylation of plasticity-related genes was determined following m^6A mRNA immunoprecipitation. Chromatin immunoprecipitation was used to examine the endogenous binding of C/EBP-α to the Fto promoter. MiR-124–mediated post-transcriptional inhibition of Fto via *C/EBPα* was determined using an in vitro model.
Results: Hippocampus of learned helplessness rats showed downregulation of Nr3c1, Creb1, and Ntrk2 along with enrichment in their m^6A methylation. A downregulation in demethylating enzyme Fto and upregulation in methylating enzyme Mettl3 were also noted. The Fto promoter was hypomethylated due to the lower expression of Dnmt1 and Dnmt3a. At the same time, there was a lower occupancy of transcription factor C/EBPα on the Fto promoter. Conversely, C/ebp-α transcript was downregulated via induced miR-124-3p expression.
Conclusions: Our study mechanistically linked defective C/EBP-α-FTO-axis, epigenetically influenced by induced expression of miR-124-3p, in modifying m^6A enrichment in plasticity-related genes. This could potentially be linked with abnormal neuronal plasticity in depression.

Keywords: M^6A Methylation, miR-124-3p, C/EBPα, FTO, depression, hippocampus, plasticity

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Significance Statement

N6-methyladenosine (m^6A) methylation is the most prevalent RNA modification in the eukaryotic system and is linked to normal brain functioning and disease states. In the present study, we examined if m^6A RNA methylation could be a missing link between the stressful environmental insults and altered expression of plasticity-related genes and if the demethylating Fto plays a key role in such modifications. In the learned helplessness model of depression, we showed higher m^6A methylation enrichment and low expression of the plasticity genes in the hippocampus. The Fto promoter was hypomethylated despite decreased expression of Dnmt1 and 3a. Also, there was a lower occupancy of transcription factor C/EBPα on the Fto promoter. We also found that stress-induced miR-124-3p was critical in reducing the LH-specific Fto expression via targeting C/ebp-α transcript. For the first time, to our knowledge, our study mechanistically linked defective miR-124-C/EBP-α-FTO-axis in modifying m^6A enrichment in plasticity-related genes in the depressed brain.

INTRODUCTION

Understanding the neurobiological complexities of major depressive disorder (MDD) is a high priority due to its debilitating impact on an individual's health and associated morbidity and mortality (Belmaker and Agam, 2008). An estimation by the World Health Organization (WHO, 2021) shows that the prevalence of MDD encompasses approximately 280 million lives worldwide. Recent epidemiological data indicated that 40.5% of mental disability-related disorders are associated with MDD (Papakostas and Ionescu, 2014). Despite the devastating impact of MDD on an individual's health, the underlying neurobiology of this disorder is still poorly understood (Nestler et al., 2002).

Impaired synaptic plasticity is a hallmark of MDD pathogenesis (Vose and Stanton, 2017; Uchida et al., 2018), often linked with modulation in gene regulatory networks (Park et al., 2006; Smalheiser, 2014). In the past decade, altered transcriptional regulation driven by DNA methylation and chromatin modifications has been actively investigated as a potential epigenetic mechanism of gene regulation in MDD besides microRNA (miRNA) regulation (Sun et al., 2013; Fass et al., 2014; Pena and Nestler, 2018; Uchida et al., 2018; Fries et al., 2019). More recently, our laboratory and others have reported that at the molecular level, dysregulation in specific miRNAs and their functions are associated with several neuropsychiatric diseases, including stressful conditions and MDD (Serafini et al., 2012; Roy et al., 2017b; Yoshino et al., 2021a). We have shown that not only are miRNAs dysregulated but they are reorganized in a manner that can give a specific phenotype. In addition, we have demonstrated that miRNAs are involved in resiliency and susceptibility to develop depression in rodents (Smalheiser et al., 2011, 2012). However, the miRNA-mediated gene expression changes are directed toward post-transcriptional level and cannot be reversed. Advancement in understanding RNA metabolism has recently added a new dimension to this diverse array of gene regulatory mechanisms (Engel and Chen, 2018; Livneh et al., 2020). Part of this comes from studies of dynamic and reversible methylation of adenosine residues of RNA species (N^6-methyladenosine [m^6A]) (Livneh et al., 2020). M^6A methylation of adenosine is conserved across eukaryotic organisms with an estimated ratio of 0.1%–0.4% in the mammalian system (Widagdo and Anggono, 2018; Livneh et al., 2020). The m^6A methylation occurs primarily on the conserved RRACH sequence motif, where R is guanine or adenine, and H is uracil, adenine, or cytosine (Zhang et al., 2020). The m^6A methylation and its regulated distribution on mammalian coding transcripts are very well orchestrated and involve the participation of 2 major writer components from the methyltransferase family, methyltransferase like-3 and 14 (METTL 3 and 14); readers of m^6A methylation YTHDF1 and 2; and a member from alpha-ketoglutarate-dependent hydroxylase subfamily, FTO (fat mass and obesity-associated protein) as demethylase (Zhang et al., 2020). The fate of the m^6A transcript could be rescued by the direct intervention of FTO as an eraser with its oxidative demethylating function (Yang et al., 2018). M^6A methylation can alter RNA metabolism by changing RNA structure, splicing, regulating mRNA maturation, promoting translation, or accelerating mRNA decay (Wang et al., 2022).

Because m^6A is reversible, dynamic, and the most prevalent type of mRNA modification in the brain, it can potentially mediate environmental stimuli-associated gene expression changes (Chang et al., 2017; Widagdo and Anggono, 2018; Yoon et al., 2018). A recent study by Engel et al. showed that restraint stress to mice decreased RNA methylation in the prefrontal cortex and increased in the amygdala (Engel and Chen, 2018; Engel et al., 2018). It was also reported that deletion of the Mettl3 or the Fto in adult neurons altered m^6A methylation-based epitranscriptome, increased fear memory, and changed the transcriptome response to fear and synaptic plasticity (Zhang et al., 2018; Spychala and Ruther, 2019).

One of the critical members of the epitranscriptomic machinery is the demethylating enzyme FTO (Yang et al., 2022). Fto knockout mice show postnatal growth retardation and reduced brain size (Li et al., 2017a). In humans, *Fto* loss of function has been associated with structural deformities in the brain, including microcephaly, growth retardation, and characteristic facial features like cleft palate (Boissel et al., 2009). Conditional knockdown of Fto in the medial prefrontal cortex vs. dorsal hippocampus had an ambivalent effect on managing memory responses (Engel et al., 2018). Importantly, Fto is critical in adult neurogenesis (Gao et al., 2020). Interestingly, a human genetic study showed a strong association of *Fto* with MDD development (Rivera et al., 2012, 2017; Liu et al., 2021). Given the critical role of FTO in gene regulation, brain functions, and behavior, it is important to examine the underlying regulatory axis that controls Fto expression and its possible role in disease pathogenesis (Liu et al., 2018).

In the present study, we tested whether m^6A RNA methylation is a missing link between the stressful environmental insults and altered expression of plasticity-related genes in MDD and whether the Fto gene plays a role in such modifications. We hypothesized that under stressful conditions, Fto, as a demethylating enzyme, will participate in modulating m^6A RNA methylation of plasticity-related genes and consequently alter their expression. We further hypothesized that the Fto promoter will be hypomethylated and there will be a lower occupancy of transcription factor C/EBPα on the Fto promoter. In addition, miR-124-3p will be critical in reducing Fto expression via targeting C/ebp-α transcript. To test this, we used the learned helplessness (LH) model of depression. Using an in vitro model system, we established an upstream regulatory axis that may control the Fto gene expression and if this regulatory mechanism is functionally impaired in LH rats. To our knowledge, this is the first study to shed light on a previously unknown regulation of the Fto gene and its possible role in MDD pathophysiology.

MATERIALS AND METHODS

Animals

The study used postnatal day 90 male Holtzman rats purchased from Envigo (Indianapolis, IN, USA). Rats were housed at 23°C and 55% humidity and were given ad libitum food and water. During acclimatization (1 week), rats were placed randomly (3/cage); however, after initial behavioral testing, they were grouped according to their behavioral phenotypes. All experiments were performed under a light cycle (8:00 AM and 10:00 AM). The protocol to induce LH behavior was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. The animal study also adhered to the international guidelines for the use and care of laboratory animals. Previous reports have shown the usefulness of Holtzman rats in modeling stress-related depression phenotype because of their higher susceptibility to developing depression under stress (Wieland et al., 1986; Padilla et al., 2010). Also, other strains of rats are either resistant to or less susceptible to this phenotype (Padilla et al., 2009). All the experiments were conducted in rats ranging from 4 to 8 per group.

Procedure to Induce LH Behavior in Rats

Figure 1A provides the paradigms used in the induction of inescapable shock (IS) and escape test . We used the same batch of animals in this study as in our earlier publication (Roy et al., 2018). Rats were given 100 random tail IS at an intensity of 1.0 mAmp for 5 seconds. The average interval between 2 shocks was 60 seconds. Escape latency (ET) was tested after 24 hours. These rats were given an additional IS on day 7 and tested for escape latency on day 8 and day 14. Another group of rats was tested for escape latency without shock, which served as control (TC). Because TC rats were handled similarly to non-learned helplessness (NLH) and LH rats, the inclusion of TC rats in the experiment helped to rule out the non-specific effects of stress caused by restraint, tail shock, or testing. The escape latency was tested using 2 trials: Fixed Ratio 1 (FR1) and 2 (FR2). In FR1 (5 trials) pretrial, rats were given a 0.6-mAmp foot shock at variable time intervals. The rats had to escape the foot shock by moving from one chamber to another without returning. In FR2 (25 trials), the rats had to cross from one compartment to another and return to the original compartment to terminate the shock. The shocks were terminated automatically after 30 seconds. Escape latencies were recorded. Based on escape latency in the FR2 trial, rats were divided into 2 groups: LH (showing escape latency ≥20 seconds) and NLH (showing escape latency <20 seconds). Generally, the rats who showed LH behavior in the FR2 trial (day 2) continued LH throughout the experimental duration (day 14). We found an almost equal distribution of rats among LH and non-LH groups. Twenty-four hours after the last ET, all rats were killed, their brains were quickly extracted, and hippocampi were dissected.

Extraction of RNA

Total RNA was extracted from the hippocampus using TRIzol-based optimized liquid-phase isolation protocol supplemented with carrier precipitation as described earlier (Roy et al., 2017a). The RNA pellet was washed twice with ice-cold 70% ethanol. After the last wash, the air-dried pellet was used to re-suspend with nuclease-free water. RNA was checked for purity by OD 260:280 ratio (NanoDrop 1000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA, USA) and running the samples on an agarose gel.

Gene Expression of m^{6}A Methylation Regulatory Enzymes and Plasticity-Related Genes

In the hippocampus of LH, NLH, and TC rats, the expression levels of Fto, Mettl3, and Mettl14 were determined by the quantitative PCR method for relative quantification of select transcript abundance method as described earlier (Roy et al., 2017a, 2020). Relative quantification of transcripts was conducted following the synthesis of the first-strand cDNA using 1 µg of total RNA. The mRNA pool was selectively reverse-transcribed using the oligo dT-based priming method. The amounts of target genes expressed were normalized to the expression of the Gapdh gene. In the hippocampus, the expression of miR-124-3p was also determined based on the cDNA preparation following poly-A tailing method (Roy et al., 2017a). Data were normalized using the U6 gene. The primer sequences for all the genes are provided in Supplemental Table 1. Fold changes between groups were measured using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_T = (C_{T\ target} - C_{T\ normalizer})_{sample} - (C_{T\ target} - C_{T\ endogenous\ gene})_{control}$ (Livak and Schmittgen, 2001).

M^{6}A Methylation Enrichment Analysis Following m^{6}A mRNA Immunoprecipitation (MeRIP)

To determine transcript-specific differential methylation enrichment, qPCR was performed with oligo primers following a MeRIP procedure. Briefly, 5 µg of total RNA was chemically fragmented following a short heat incubation. Afterward, 10% of fragmented RNA was reserved as input for reverse transcription. Full-length m^{6}A-tagged transcripts were immunoprecipitated using a rabbit polyclonal anti-m^{6}A antibody (Synaptic Systems, Goettingen, Germany) conjugated with protein G Dynabeads (ThermoFisher Scientific, DE, USA). Reverse transcription of input RNA and m^{6}A antibody pull-down RNA was performed using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). The relative abundance of expressed gene transcripts and immune-enriched m^{6}A transcripts was measured with a quantitative real-time PCR machine (Stratagene MxPro3005, Santa Clara, CA, USA) using EvaGreen chemistry (Applied Biological Material Inc, Richmond, Canada). The relative enrichment of m^{6}A methylation on the select gene transcripts was quantified after normalization with the input samples. Fold-change values were determined following Livak's ΔΔCt method (Livak and Schmittgen, 2001). The oligo sequences used for MeRIP-qPCR are mentioned in supplemental Table 1.

Chromatin Immunoprecipitation (ChIP)-qPCR–Based in Vivo Binding of C/EBP-α on Fto Promoter

Following the ChIP methods described in our previous publication (Ludwig et al., 2019), we determined the endogenous binding of C/EBP-α to the proximal element present on the upstream region of rat Fto promoter using C/EBP-α antibody (C/EBP-α, 14AA; Santa Cruz, TX, USA)-mediated immune pull-down. Relative enrichment of C/EBP-α transcription factor binding to the Fto promoter was detected by qPCR using primer pairs specific to rat Fto promoter (supplemental Table 1).

In Vitro Cellular Transfection Assay

An in vitro cellular model was used to achieve an miRNA-mediated post-transcriptional inhibition of specific downstream genes. For this, SHSY5Y neuroblastoma cells were transfected (SHSY5Y ATCC CRL2266) with double-stranded RNA oligos (Dharmacon, Lafayette, USA), mimicking endogenous miR-124-3p (Mimic-124) and antisense of miR-124-3p (Anti-124) using Lipofectamine RNAiMAX (Invitrogen, NY, USA). The results were compared with the control cell line group similarly transfected with scramble oligo. In a separate set of experiments, the same SHSY5Y neuroblastoma cells (SHSY5Y ATCC CRL2266) were transfected with uniquely modified 2'-deoxy-2'-fluoro-arabinoguanosine antisense oligonucleotides (Aum Biotech, Philadelphia, PA, USA), mimicking endogenous miR-124-3p (Mimic-124) and antisense of miR-124-3p (Anti-124). The results were compared with the control cell line group similarly transfected with scramble oligo. After an incubation period of 48 hours at 37°C in a 5% CO_2 atmosphere, the transfected cells were harvested to collect chromatin lysate for a further downstream ChIP experiment.

ChIP-qPCR–Based in Vitro Transcriptional Analysis of FTO Gene Promoter via C/EBPα Binding

The influence of miR-124-3p inhibition on the binding of C/EBPα transcription factor to *FTO* gene promoter was studied following a C/EBPα-specific antibody (Santacruz Biotechnology)-mediated ChIP experiment. miRNA oligo transfected cellular lysates were used to prepare chromatin fractions. The harvested cells were used for 1% formaldehyde-based chemical cross-linking for 15 minutes at room temperature. The cross-linking reaction was quenched by adding 125 mM glycine with an additional incubation period of 5 minutes at room temperature. The cross-linked homogenates were washed twice with ice-cold phosphate buffer saline and lysed with ice-cold cell lysis buffer supplemented with protease and proteasomal inhibitors for 15 minutes on an ice bath. The lysed suspension was again homogenized to avoid cellular clumps and subsequently centrifuged to decant out any extracellular debris. Finally, chromatin was solubilized and extracted by incubating on ice for 30 minutes with protease and proteasomal inhibitors supplemented with nuclear lysis buffer. The released chromatin fraction was sonicated to obtain chromatin fragments of 200–600 bp. Insoluble materials from sheared chromatin were cleared with centrifugation at 14k rpm for 20 minutes. An equal amount of diluted chromatin samples devoid of 10% fraction (input) was used in immunoprecipitation with C/EBPα (Abcam, MA, USA) pre-conjugated protein A/G magnetic beads for an overnight period. Immunoenriched chromatin-bead complex was washed sequentially with low salt buffer twice, high salt buffer once, LiCl buffer once, and TE buffer twice. After washing, the DNA-protein complex was uncoupled from the beads using freshly prepared elution buffer and reverse cross-linked at 65°C for 4 hours with vigorous shaking. Similar steps were followed for preparing input fraction DNA. The immunoprecipitated and input fraction DNAs were then purified using the phenol:chloroform:isoamyl alcohol method. Finally, immunoprecipitated DNAs were subjected to relative quantification with EvaGreen Dye (Applied Biological Material Inc., BC, Canada)-based chemistry. Amplification in the qPCR system was conducted using DNA samples collected after immunoprecipitation and input control. The primers used for amplifying the *FTO* promoter element are provided in supplemental Table 1.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) was used for all the data analysis. The data are represented as mean ± SEM. TC, LH, and non-LH groups were compared using 1-way ANOVA. Post-hoc comparisons were calculated by Tukey's method of multiple comparisons. TC and LH groups were compared using independent sample equal variance *t* tests. The significance level was set at $P \leq .05$.

RESULTS

Escape Latencies

Based on escape latencies, the rats were divided into LH (vulnerable to stress-induced depression), NLH (resilience to depression after receiving similar stress stimuli), and TC (no shock but tested for escape latency) groups. As shown in Figure 1B, significant differences in escape latencies ($P < .001$) between TC, NLH, and LH groups were observed when tested on days 2 (df = 3; $F = 147.9$; $P < .001$), 8 (df = 3; $F = 104.8$; $P < .001$), and 14 (df = 3; $F = 216.6$; $P < .001$). Individual group comparisons showed significantly higher escape latencies ($P < .001$) for LH rats compared with TC and NLH rats on days 2, 8, and 14. NLH rats did not show any significant differences in escape latencies compared with the TC group at any time point. Rats with LH or NLH behavior on days 2 and 7 showed the same behavior when tested on day 14.

Expression of m6A Methylation and Demethylation Enzymes in the Hippocampus of LH Rats

The expression levels of Fto, Mettl3, and Mettl14 genes were examined in the hippocampus of LH rats (Figure 1C). We found significant changes in the expression of Fto and Mettl3 genes. When compared with TC, the LH group of rats showed a highly significant ($P = .005$) expression downregulation (approximately 82%) of the Fto gene. On the contrary, Mettl3 was significantly ($P = .031$) upregulated (approximately 38%) in LH rats. No significant ($P = .06$) change in Mettl14 expression was noted when LH rats were compared with TC rats. No significant differences were observed in the expression of Fto ($P = .38$)., Mettl3 ($P = .06$), and Mettl14 ($P = .82$) between TC and NLH rats.

Because the expression of m^6A-methylating and -demethylating enzymes was restricted to LH rats, all subsequent experiments were performed in the hippocampus of LH and TC rats.

Altered m6A-Based RNA Methylation of Plasticity Genes Following MeRIP-qPCR in the Hippocampus of LH Rats

We examined the m^6A methylation status of select plasticity-related genes (Nr3c1, Creb1, Bdnf, and Ntrk2) in the hippocampus of LH and TC rats (Figure 2A–D). Methylation-associated changes were investigated following an m^6A-specific antibody-based MeRIP qPCR. MeRIP-qPCR data showed significant methylation enrichment of 3 select gene transcripts—Nr3c1, Creb1, and Ntrk2—in LH rats. In the LH group, the largest methylation enrichment was noticed for the Nr3c1 gene (approximately 20%), which was highly significant ($P < .05$). A similar high-fold enrichment (20%) was noted for the Creb1 transcript, and this change was also highly significant ($P < .05$). The Ntrk2 transcript also demonstrated significant ($P = .04$) enrichment (approximately fourfold). Contrary to

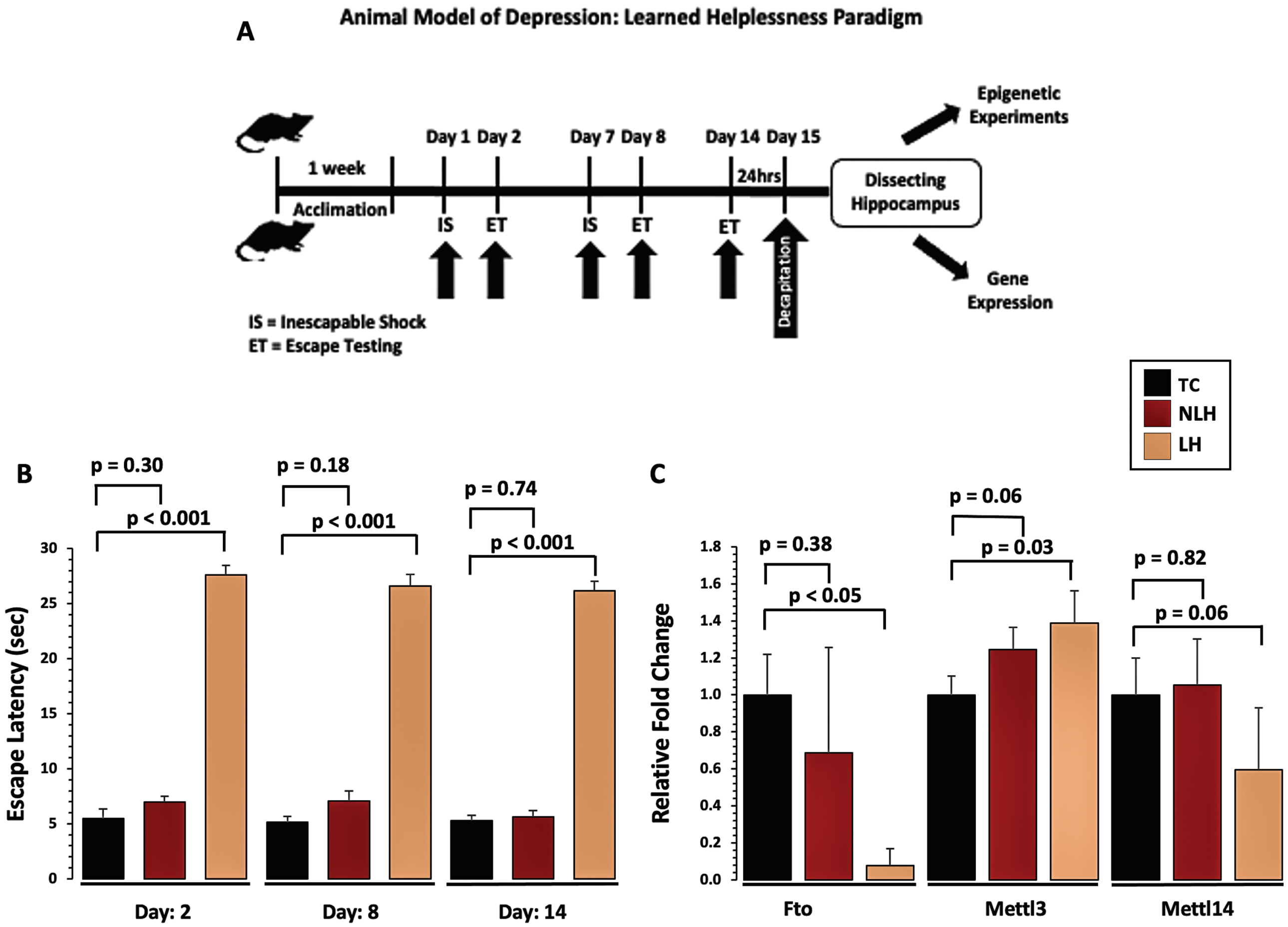

Figure 1. Induction of learned helplessness (LH) behavior and changes in expression of genes associated with epitranscriptomic regulation in the hippocampus of LH rats. (A) Schematic diagram of the timeline followed as part of the stress paradigm to induce LH behavior in rats. Rats were given 100 random tail inescapable shocks (IS) at an intensity of 1.0 mAmp for 5 seconds. The average interval between 2 shocks was 60 seconds. Escape latency (ET) was tested after 24 hours. These rats were given an additional IS on day 7 and tested for ET on day 8 and again on day 14. Based on escape latency, rats were divided into 2 groups: LH (showing ET ≥20 seconds) and non-learned helplessness (NLH, showing escape latency <20 seconds). Another group of rats was tested for ET without giving any shock and served as control (TC). Twenty-four hours after the last ET, all rats were killed, brains were quickly extracted, and hippocampi were dissected. (B) Mean ETs in NLH and LH rats compared with TC rats. Bar diagrams represent ETs in TC, NLH, and LH rats measured on days 2, 8, and 14. Data are the mean ± SEM. Overall group comparisons are as follows: day 2: df = 3, F = 147.95; day 8: df = 3, F = 104.89; and day 14: df = 3, F = 216.68. The bar diagrams represent ETs in TC and NLH rats measured on days 2, 8, and 14. Data are the mean ± SEM. On day 2, the NLH rats did not show any significant (*P* = .30) difference in ET compared with the TC group. A significantly (*P* <.001) higher ET was observed for LH rats compared with TC on day 2. Similarly, LH rats showed a significant difference (*P* <.001) in mean ET compared with the NLH group on day 2. On day 8, NLH rats showed no significant (*P* = .18) difference in ET compared with the TC group. A significantly (*P* < .001) higher ET was noted for LH rats compared with TC rats. On day 14, NLH rats did not show any significant (*P* = .744) difference in ET compared with the TC group. Individual group comparison identified a significantly (*P* < .001) higher ET for LH rats compared with TC rats. (C) Gene expression changes in m^6A methylation-processing enzymes. Significant (*P* <.05) expression downregulation (approximately 82%) was found for the Fto gene in LH rats (n = 3) compared with TC rats (n = 8). No significant differences (*P* = .38) were observed in the expression of Fto in NLH rats (n = 8) compared with TC rats (n = 8). Mettl3 was significantly (*P* = .031) upregulated (approximately 38%) in LH rats (n = 5) compared with TC rats (n = 7). No significant (*P* = .06) changes were noted for Mettl3 in NLH (n = 7) rats compared with TC rats (n = 8). No significant differences were noted in Mettl14 expression in TC rats (n = 8) compared independently between LH (n = 8) and NLH (n = 8) rats (*P* = .06 for LH and *P* = .82 for NLH). Data are the mean ±SEM. Relative fold changes were determined based on Gapdh normalization following the ΔΔCT method.

the other 3 genes, m^6A methylation enrichment was significantly (*P* < .05) lower for the Bdnf transcript in the LH group compared with the TC group.

Expression of Plasticity Genes by qPCR in the Hippocampus of LH Rats

To examine if changes in m^6A methylation were related to changes in the expression of the plasticity-related genes, we determined the expression of Nr3c1, Creb1, Bdnf, and Ntrk2 genes (Figure 2E–H) in the hippocampus of the same LH and TC rats in which m^6A methylation was examined. We found that the expression levels of Nr3c1 (*P* = .003), Creb1 (*P* = .01), Ntrk2 (*P* = .01), and Bdnf (*P* = .05) were significantly downregulated in LH rats compared with TC rats.

In Silico Mapping and DNA-Based Methylation Patterns of Fto Gene Promoter

We further examined the underlying regulatory mechanisms behind altered Fto expression in LH rats. Our bioinformatic analysis helped us map rats' upstream regulatory elements of the Fto gene (Figure 3A). As shown in the schematic diagram (Figure 3A), Fto has 2 exons enriched with CpG sites immediately downstream of the transcription start site (TSS) and a C/EBP-α binding motif in between. Promoter-specific methylation

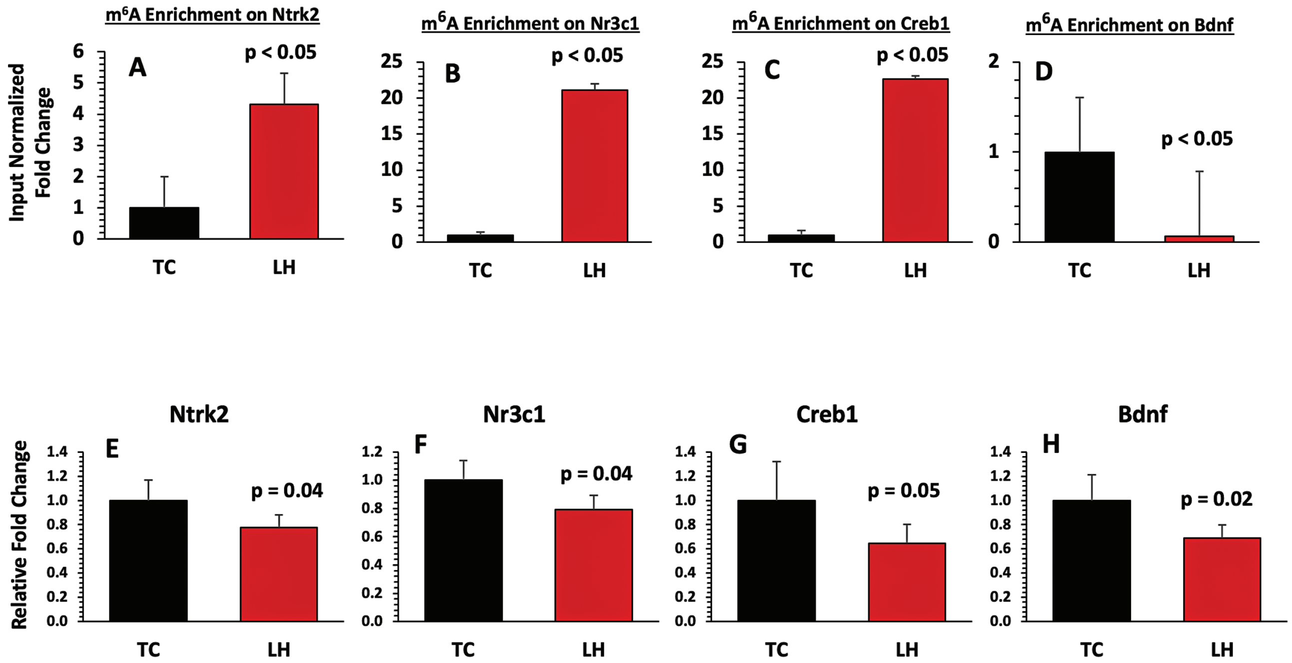

Figure 2. N6-methyladenosine (M^{6}A) enrichment and expression levels of select plasticity-related transcripts in the hippocampus of learned helplessness (LH) rats. (A) Significantly increased m^{6}A levels in the 3' untranslated region (UTR) of Ntrk2 transcript compared between control (TC) (n = 5) and LH (n = 5) rats, (P < .05). (B) Significantly increased m^{6}A levels in the 3'UTR of Nr3c1 transcript compared between TC (n = 4) and LH (n = 5) rats (P < .05). (C) Significantly increased m^{6}A levels in the 3'UTR of Creb1 transcript compared between TC (n = 5) and LH (n = 4) rats (P < .05). (D) Significantly decreased m^{6}A levels in the 3'UTR of Bdnf transcript compared between TC (n = 5) and LH (n = 4) rats (P < .05). (E) Bar diagram showing highly significant (P = .04) expression downregulation (approximately 82%) of the Ntrk2 gene in LH rats (n = 3) compared with TC (n = 8). (F) Significant downregulation (approximately 38%) of Nr3c1 gene (P = .04) in LH (n = 5) compared with TC (n = 7) rats. (G) Bar diagram demonstrating significant expression increase for Creb1 gene in LH (n = 8) compared with TC (n = 8) rats. (H) LH (n = 5) rats show significant (P = .02) decrease in Bdnf gene expression compared with TC (n = 7) rats. Data are the mean ±SEM. For MeRIP-qPCR, relative 3′ UTR enrichment was determined based on 10% input normalization following qPCR. For gene expression, relative fold change was determined based on Gapdh normalization following the ΔΔCT method.

changes are epigenetically essential to regulate gene transcription. We asked whether changes related to Fto gene expression were linked with promoter-specific methylation in LH rats. Figure 3B shows an Fto promoter CpG island map within 1 kb upstream of the rat Fto gene. We measured 5-mC enrichment of those mapped CpG sites on the Fto promoter to understand if LH-specific Fto expression repression is contributed by promoter hypermethylation. Our methylation-specific MeDIP followed by qPCR showed a significant hypomethylation (30% less enriched methylation; P = .04) of Fto gene promoter in the hippocampus of LH rats compared with TC rats (Figure 3C).

Expression of DNA Methylation Enzymes in the Hippocampus of LH Rats

To examine if promoter hypomethylation of the Fto gene was associated with altered expression of DNA methylation enzymes, we tested the transcript levels of DNA methyltransferase (Dnmt)1, Dnmt3a, and Dnmt3b. We found that the expression of Dnmt1 and Dnmt3a was significantly downregulated (Dnmt1, approximately 27%, P = .02; Dnmt3a, approximately 23%, P = .05) in the hippocampus of LH rats (Figure 3D–E) without any changes in Dnmt3b (data not shown).

Expression Variability of Transcription Factor C/EBP-α in the Hippocampus of LH Rats

Next, we sought to examine if the changes in Fto expression were transcriptionally related to transcription factor C/EBP-α. C/EBP-α (CCAAT enhancer-binding protein) acts as an early response transcription factor and is suggested to have an inducing effect on Fto gene transcription. We found a significant (P = .003) decrease (approximately 44%) in C/ebp-α gene expression in LH rats compared with TC rats (Figure 3F).

In Vivo Binding of CEBP-α on Fto Gene Promoter

As shown in the schematic diagram in Figure 3A, Fto has 2 exons enriched with CpG sites immediately downstream of the TSS. Besides, an evolutionarily conserved C/EBP-α binding site was also mapped right after exon 1. To determine the regulatory effect of C/EBP-α on Fto gene expression, we performed a ChIP assay using chromatin lysate collected from rat hippocampus. The results showed (Figure 3G) significantly decreased (approximately 40%) binding of C/EBP-α on Fto promoter in the hippocampus of LH rats (P = .05).

Expression of miR-124-3p in the Hippocampus of LH Rats

We sought to determine if any changes are associated with miR-124-3p expression in the hippocampus of LH rats, given that C/ebp-α is a direct target of miR-124-3p. Our qPCR assay based on miR-124-3p specific primer determined a significant (P = .01) increase (30%) in expression in LH rats compared with TC rats (Figure 3H). However, no significant (P = .23) difference was noted in NLH rats compared with TC rats.

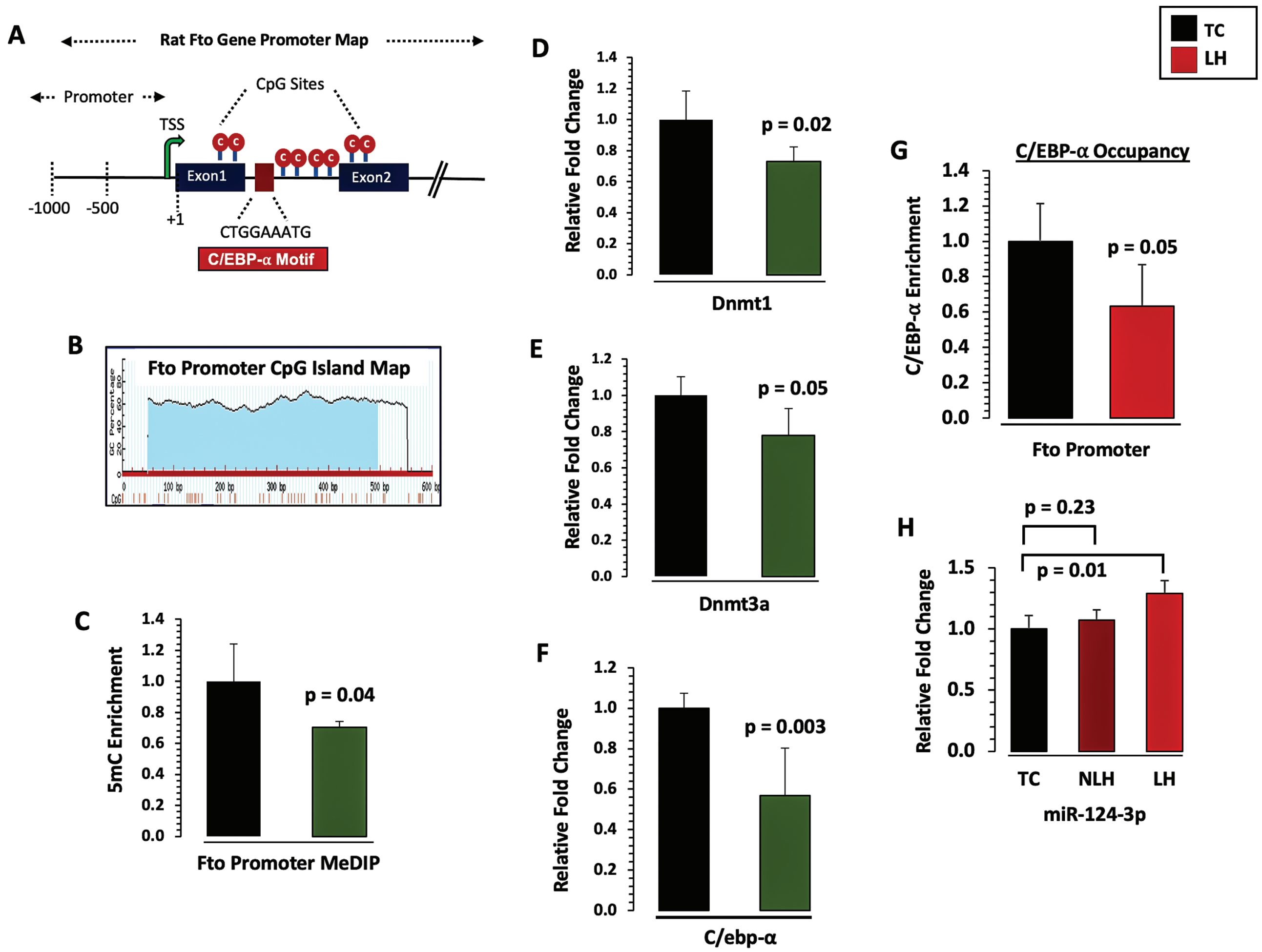

Figure 3. Fto gene promoter regulation following DNA methylation changes and binding of transcription factor C/EBP-α in the hippocampus of learned helplessness (LH) rats. (A) Schematic diagram showing immediate upstream (1000 bp) and downstream promoter region of Fto from the transcription start site (TSS). The diagram also shows the relative binding site for C/EBP-α, highlighting the consensus motif sequencing flanked by exon 1 and 2 of the Fto gene. The in silico prediction of a stretch of CpG sites is indicated with ball and stick drawing spanning across the first and second exons of the Fto gene. (B) In silico prediction and mapping of a potential CpG island on Fto gene promoter. (C) Bar diagram showing significant ($P = .04$) decrease in DNA methylation (30%) of the Fto gene promoter in LH ($n = 7$) compared with control (TC; $n = 4$) rats. (D–E) The graphs represent significant expression downregulation of Dnmt1 and Dnmt3a genes (Dnmt1, approximately 27%, $P = .02$; Dnmt3a, approximately 23%, $P = .05$) in LH rats compared with TC rats. (F) Significant ($P = .003$) downregulation of C/EBP-α expression (50%) in LH rats ($n = 6$) compared with TC rats ($n = 3$). (G) Chromatin immunoprecipitation (ChIP) assay for C/EBP-α binding on the Fto promoter. Input normalized fold-change values were determined in the LH rats compared with TC rats. Binding of C/EBP-α on Fto gene promoter demonstrated the 40% lower promoter occupancy ($P = .05$) in LH rats compared with TC rats ($n = 3$/group). (H) In vivo expression profile of miR-124-3p in LH ($n = 7$) rats compared with TC rats ($n = 7$). Relative transcript abundance of mature miR-124-3p in LH rat hippocampus showed an approximately 30% increase ($P = .01$) compared with TC. The Gapdh gene was used as a normalizer for gene expression assays, and miRNA expression data were normalized to U6 expression. All values are represented as ± SEM.

In Vitro Gene Expression Changes in miR-124-3p–Transfected SHSY5Y Cells

Previous reports have suggested that *C/EBPα* is a direct target of miR-124-3p (Ponomarev et al., 2011). We induced the expression of miR-124-3p in vitro by oligo transfection to examine its effect on *C/EBPα* gene expression (Figure 4A). Transfection of SHSY5Y cells with mimic and antisense oligo against miR-124-3p resulted in significant expression variability of *C/EBPα* transcripts. We found a significant ($P = .03$) decrease (50%) in the expression of the *C/EBPα* gene in the miR-124-3p mimic transfection group compared with the scrambled control (Figure 4B). Interestingly, the depleted expression of *C/EBPα* in the mimic group was significantly ($P = .003$) restored in the miR124-3p anti-oligo–transfected group. In the same transfection group, we tested the expression of *FTO*, *METTL3*, and *METTL14* genes. We found that none of the enzymes showed significant differences in the miR-124-3p over-or underexpression group (Figure 4C–E).

Binding of C/EBPα on *FTO* Gene Promoter in miR-124-3p Oligo–Transfected SHSY5Y Cells

In the SHSY5Y cell line, we determined qChIP-based binding of C/EBPα transcription factor on *FTO* gene promoter under miR-124-3p regulation. Our qPCR-based ChIP assay showed significant differences in C/EBPα binding to the *FTO* gene promoter under miR-124-3p over- and underexpression. A significant ($P = .01$) reduction (approximately 23%) in promoter occupancy was noted when the scramble oligo-transfected control group was compared with the mimic-124-3p–transfected overexpression group. MiR-124-3p knockdown further reduced (approximately

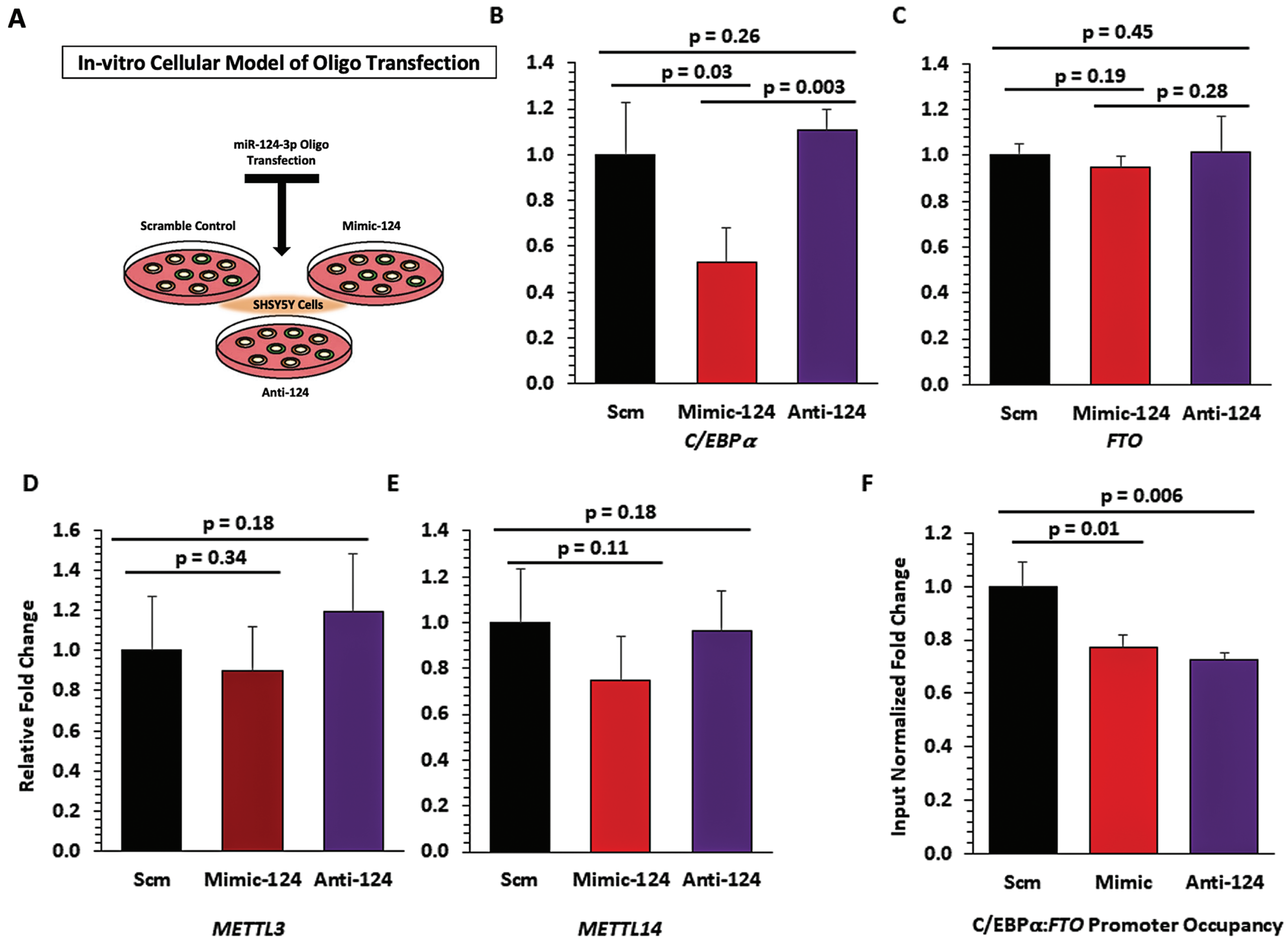

Figure 4. Expression profiling of N6-methyladenosine (M⁶A) methylation genes in response to miR-124-3p overexpression using the SHSY5Y cellular model. (A) Schematic diagram of in vitro study showing miRNA oligo transfection strategy. (B–E) Bar diagrams representing the relative expression of *C/EBPα*, *FTO*, *METTL3*, and *METTL14* transcripts in SHSY5Y cell line transiently transfected with mimic miR-124-3p overexpression oligo (mimic-124) or anti-miR-124-3p overexpression oligo (anti-124) and compared with control cells. The expression level of C/EBPα in the SHSY5Y cell line was individually transfected with miR-124-3p oligo (mimic-124) and miR-124-3p anti-sense oligo (anti-124). Compared with the control group, the mimic-124 transfected cell line showed a significant (P = .03) decrease (52%) in *C/EBPα* expression, whereas an increased expression of *C/EBPα* was observed in the anti-124–transfected group compared with the control. A significant increase was noted for *C/EBPα* in the anti-miR-124–transfected group compared independently with mimic-miR-124. The other 3 genes—*FTO*, *METTL3*, and *METTL14*—did not show any significant changes when the control group was independently compared with mimic-124 and anti-124 groups. (F) Chromatin immunoprecipitation (ChIP) assay for C/EBPα binding on the Fto promoter in the SHSY5Y cell line transiently transfected with mimic miR-124-3p overexpression oligo (mimic-124) or anti-miR-124-3p overexpression oligo (anti-124) and compared with control cells. Input normalized fold-change values were determined for C/EBPα binding on the Fto gene promoter. The binding of C/EBPα on the Fto gene promoter demonstrated significantly (P = .01) low promoter occupancy (23%) in the mimic-124–transfected cell group compared with the control. The anti-miR-124t–transfected group exhibited significantly (P = .006) less binding (28%) of C/EBPα on *FTO* promoter compared with control. *GAPDH* was used as a normalizer for gene expression assays. Values are the mean ± SEM.

28%; P = .006) the binding of C/EBPα on the *FTO* gene promoter compared with the scramble group (Figure 4F).

DISCUSSION

In this study, using an LH rat model of depression, we showed higher m⁶A methylation enrichment and low expression of plasticity genes in the hippocampus. The Fto promoter was hypomethylated despite decreased expression of Dnmt1 and 3a. Also, there was a lower occupancy of transcription factor C/EBPα on the Fto promoter. We also found that stress-induced miR-124-3p regulated Fto expression via targeting C/ebp-α transcript.

The LH rat model is considered one of the highly reliable animal models of depression (Maier, 1984; Vollmayr and Henn, 2001). It represents the theoretical basis of the origin and development of depression and is a combination of cognitive and neurovegetative abnormalities and genetic susceptibility (Jesberger and Richardson, 1985; Vollmayr and Henn, 2001; Nestler et al., 2002). In the past, we have successfully used this model in delineating neurobiological changes associated with stress-induced depression (Roy et al., 2018). We chose to examine the hippocampus, because this brain region is closely associated with learning, memory, and emotions, and depression is associated with decreased hippocampal synaptic plasticity and neuronal atrophy (Howland and Wang, 2008; Kim et al., 2015). In addition, several imaging studies suggest structural abnormalities in the hippocampus of depressed patients (Sheline et al., 1996; Frodl et al., 2002, 2006).

We observed a clear behavioral differentiation between LH and NLH rats because the escape latency in LH rats differed significantly from that of NLH and TC rats. Whereas NLH rats showed an escape latency similar to that of TC rats, the escape latency of LH rats was significantly higher than that of NLH and TC rats.

In the hippocampus of LH rats, we found that mRNA expression of plasticity-related genes Ntrk1, Creb1, Nr3c1, and Bdnf was significantly downregulated compared with TC and NLH rats. To test whether m^{6}A-based epitranscriptomic mechanisms could be involved in gene regulation, using a specific m^{6}A antibody and MeRIP-qPCR technique, we examined m^{6}A methylation enrichment in the plasticity-related genes that were downregulated in LH rats. We found that the hippocampus of LH rats had many-fold higher m^{6}A enrichment in all these genes except Bdnf. M^{6}A methylation enrichment of the 3 transcripts presents an inverse relationship with their expression profile. The contrasting patterns of gene expression and m^{6}A methylation explain the influence of m^{6}A methylation regulation on transcript instability. In contrast to other genes, the Bdnf gene showed both lower expression and m^{6}A methylation. This suggests that m^{6}A methylation and consequent post-transcriptional modification are gene specific.

Reversible m^{6}A methylation modifications and their regulated distribution on mammalian transcriptome is a well-orchestrated phenomenon (Dominissini et al., 2016; Wang et al., 2016, 2022; Ivanova et al., 2017). Upon receiving extracellular environmental stimuli, the writer methyltransferases (METTL3 and METTL14) are recruited at cognate m^{6}A consensus sequences selectively present on 3'untranslated region (UTR) of mammalian mRNA molecules, leading to their preferential binding with the reader protein YTHDF2 (Du et al., 2016; Li et al., 2018). Successful interaction of this reader molecule with the m^{6}A mark finalizes the subcellular localization of methylated transcripts at cytosolic P-bodies with a catabolic fate (Du et al., 2016). However, the assigned fate of m^{6}A transcripts is rescued by the direct intervention of FTO as an eraser with its oxidative demethylating function (Walters et al., 2017). In this study, we found that the expression of the Fto gene was significantly downregulated in the hippocampus of LH rats. Because the established role of the Fto gene necessarily implies the phenomena of RNA transcript demethylation, our current observation of the reduced Fto gene indicates its compromised functionality in stripping off the methyl group from the transcripts in the hippocampus of LH rats.

In addition to FTO, we found a significant expression upregulation in the Mettl3 gene without any change in the expression of Mettl14 in the hippocampus of LH rats. This observation suggests that METTL3 drives active methylation status in the hippocampus of LH rats. METTL3 is enzymatically active in a multiprotein complex as a heterodimer with METTL14 to transfer methyl group on a consensus sequence motif (GAC) preferentially localized in 3'UTR (Yang et al., 2018; Lee et al., 2020). No significant change in METTL14 and a higher abundance of METTLl3 suggest the availability of more heterodimers to enhance the methyl group transfer on adenosine residue in LH rats. The effect of this regulated RNA methylation on putative transcripts with the m^{6}A methylation consensus motif was further substantiated by our findings of higher m^{6}A methylation in Ntrk1, Creb1, and Nr3c1 transcripts.

To understand how the Fto gene is regulated in LH rats, we mapped CpG sites on the Fto promoter and found that Fto has 2 exons enriched with CpG sites immediately downstream of the TSS. Our examination of MeDIP followed by qPCR showed a significant hypomethylation of Fto gene. To examine if the promoter hypomethylation of the Fto gene was associated with the altered expression of DNA methyltransferases, we determined the expression levels of Dnmts. We found that the levels of Dnmt1 and Dnmt3a were significantly downregulated. Our data suggest that hypomethylation of the Fto gene could be associated with lower expression of select Dnmts in LH rats. Interestingly, despite lower promoter methylation and lower expression of Dnmt1 and Dnmt3a expression, te hFto gene was downregulated in LH rats. To further examine the mechanism of Fto gene regulation, we mapped the 2 exons of the Fto gene, which had enriched CpG sites, and found a rich C/EBP-α binding site right after exon 1. Under normal conditions, C/EBP-α acts as a transcription factor to functionally induce the expression of the Fto gene (Ren et al., 2014). In LH rats, we found that not only was the expression of C/EBP-α lower, but there was a suboptimal C/EBP-α occupancy on the promoter of the Fto gene. This raises an exciting possibility that despite lower promoter methylation, less occupancy of C/EBP-α on the Fto promoter could lead to Fto downregulation.

MiRNAs are one of the critical epigenetic modifiers that belong to the non-coding RNA family, with a specific epigenetic role in modulating the coding potential of transcribed mRNAs based on characteristic sequence complementarity (Dwivedi, 2011; Roy et al., 2017a). miRNAs regulate the translation of proteins (Schratt, 2009) and participate in the altered gene expression that accompanies long-term potentiation (Park et al., 2006) and learning and memory (Smalheiser et al., 2010). We and other investigators have repeatedly shown the involvement of miRNAs in stress-related disorders, including major depression (Dwivedi, 2014; Lopizzo et al., 2019). MiR-124 has emerged as a prominent miRNA that participates in stress-related disorders (Roy et al., 2017a; Gu et al., 2019; Yang et al., 2020). Interestingly, the miR-124 variant miR-124-3p directly targets *C/EBPα* and regulates its expression (Yu et al., 2017). We found a significant upregulation of miR-124-3p in the LH rat hippocampus. Our in vitro data using a neuroblastoma cell line suggested a mechanistic role of miR-124-3p in regulating the *FTO* gene. Whereas we did not find a direct role of miR-124 in regulating the expression of m^{6}A-methylating and -demethylating enzymes *FTO*, *METTL3*, and *METTL14*, in vitro transfection of miR-124 mimic dramatically inhibited the expression of *C/EBPα*. This inhibition was rescued in the miR-124-3p anti-oligo–transfected group. These data suggest that miR-124-3p directly regulates the expression of *C/EBPα*. Based on our findings, we propose that an impaired epitranscriptomic regulation of plasticity-related genes in the hippocampus of LH rats might be mechanistically linked to defective transcription factor C/EBP-α-Fto axis, which is epigenetically influenced by induced expression of miR-124-3p under stressful conditions (Figure 5).

Our results are functionally relevant MDD-associated impaired synaptic plasticity involving dynamic changes in gene regulatory networks (Bristot et al., 2020; Yoshino et al., 2021b). These dynamic changes quickly respond to environmental stimuli (Lopizzo et al., 2019). With the recent advent of epitranscriptomic modification, such dynamism in gene regulation may occur in concert with m^{6}A RNA methylation, the most prevalent type of epitranscriptomic modification in the brain (Chang et al., 2017). In this context, it is important to note that FTO plays a significant role not only in the processing of m^{6}A enrichment on a gene, but it has a direct role in brain development and function (Widagdo and Anggono, 2018; Chang et al., 2022). The *FTO* gene is abundantly expressed in the brain and participates in neurogenesis (Li et al., 2017b; Gao et al., 2020; Du et al., 2021) and memory deficits (Walters et al., 2017; Spychala and Ruther, 2019; Leonetti et al., 2020). Whereas contextual fear conditioning reduces Fto expression in dendrites and dendritic spines of mouse dorsal hippocampus (Walters et al., 2017), Fto knockdown in the medial prefrontal cortex enhances consolidated fear memory with an increase in m^{6}A methylation at specific gene transcripts (Widagdo et al., 2016; Engel et al., 2018).

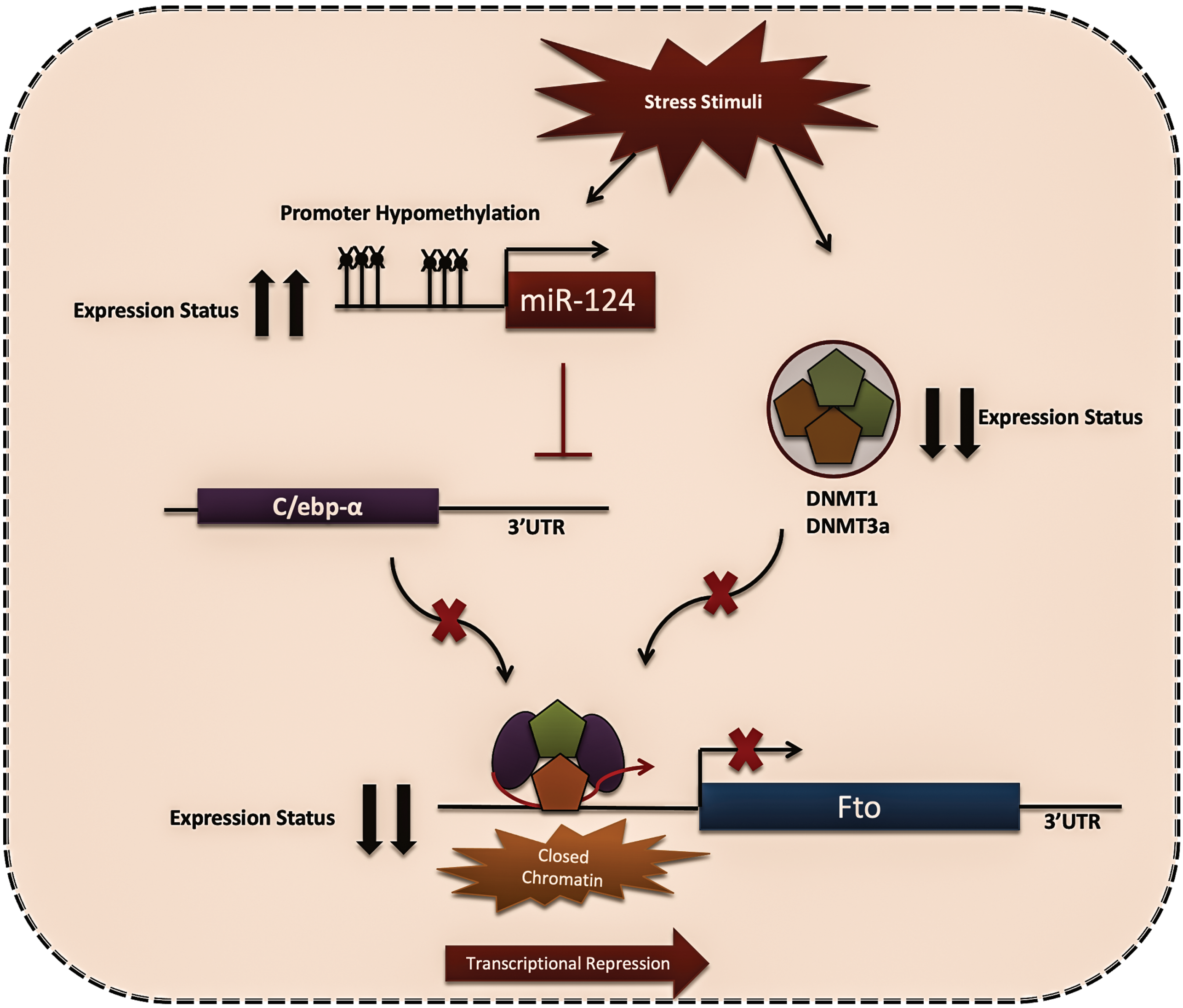

Figure 5. A cellular model of Fto gene inhibition mediated via miR-124-3p- C/EBP-α axis under the stress-induced maladaptive changes in the hippocampus of learned helplessness (LH) rats. We presume that the low expression level of the Fto gene in the hippocampus of depressed rats may not be related to its induced methylation status despite the presence of a strong CpG island on its promoter. Instead, a promoter hypomethylation of the Fto gene is achieved due to the low expression level of DNMTs. On the contrary, the stress-induced expression of miR-124-3p could be responsible for the expression downregulation of the Fto gene due to the inhibition of transcription factor C/EBP-α, directly targeted by miR-124-3p. Altogether, we propose a hypothesis that an impaired epitranscriptomic regulation of plasticity-related genes in the hippocampus of LH rats might be mechanistically linked to defective transcription factor C/EBP-α-Fto axis, which is epigenetically influenced by induced expression of miR-124-3p under depressive conditions.

The inactivation of FTO impairs dopamine receptor type 2 and its depletion in the midbrain and striatum cause increased adenosine methylation in a subset of mRNAs associated with signaling pathways regulating learning, reward behavior, motor functions, and feeding in mice (Hess et al., 2013). Interestingly, the *FTO* gene has been reported to be a polymorphic candidate for MDD (Rivera et al., 2012, 2017). Additionally, Fto knockout mice show increased corticosterone levels in plasma (Spychala and Ruther, 2019). Moreover, FTO positively regulates mTOR signaling, which is involved in depression pathophysiology and the mechanism of ketamine action (Li et al., 2010).

Our findings of the involvement of miR-124 in regulating the FTO gene via transcription factor C/EBP-α are highly relevant. We and other investigators have shown that miR-124-3p plays a crucial role in synaptic plasticity and stress-related disorders (Sun et al., 2015; Dwivedi, 2017; Roy et al., 2017a; Gu et al., 2019; Yang et al., 2020; Zeng et al., 2021). Knockdown of miR-124 reduces depression-like behavior in chronic unpredictable stress–induced depressive rats (Yang et al., 2020). Our present study shows concerted epigenetic modifications mediated by FTO and Mettl3 and regulation of the *Fto* gene by miR-124 and *C/EBPα* in modulating the expression of plasticity-related genes and, consequently, depressive behavior.

The study has some limitations. For example, the present study used specific plasticity-related genes. Large-scale transcriptome-wide modifications in FTO-mediated m^{6}A methylation could provide a broader role in m^{6}A methylation in depressive behavior. M^{6}A methylation is brain-region specific and corresponds to the variability in biological functions (Chang et al., 2017). Thus, other brain areas also need to be studied. Additionally, the impact of sex on methylation has not been determined in this study. Sex-specific changes at the methylation level and Fto modulation can provide more insights into their role in MDD pathogenesis. Also, it will be interesting to see if similar changes occur in the postmortem brain of MDD patients and whether these changes are specific to depression or also occur in other psychiatric disorders.

Altogether, our study, for the first time to our knowledge, highlights the role of demethylase Fto in regulating m^{6}A methylation under chronic stress and opens up a new vista to examine m^{6}A methylation/Fto-based molecular circuitry in depression pathophysiology. The epitranscriptomic mechanism of gene regulation is a fascinating yet unexplored area in disease pathogenesis. Because many genes contain m^{6}A sites and only a majority are un- or partially methylated, a large dynamic margin exists for m^{6}A-based regulation across the transcriptome. This offers a unique opportunity for m^{6}A methylation sites to allow a rapidly tuned response to external stimuli, which could be critical in developing depression. In addition, depression pathophysiology involves alterations in gene network(s) (Bagot et al., 2016; Gerring et al., 2019; Geng and Huang, 2021). Epitranscriptomic modifications and subsequent changes in gene expression could significantly participate in such gene network changes. Overall, epitranscriptomic regulation of genes can be used as a novel "molecular tool" not only to understand the etiopathogenesis of depressive behavior but also to generate new molecular-based therapies to treat this severe mental disorder.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Conflict of Interest

None.

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