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Object-Location Training Elicits an Overlapping but Temporally Distinct Transcriptional Profile from Contextual Fear Conditioning

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Abstract

Hippocampus-dependent learning is known to induce changes in gene expression, but information on gene expression differences between different learning paradigms that require the hippocampus is limited. The bulk of studies investigating RNA expression after learning use the contextual fear conditioning task, which couples a novel environment with a footshock. Although contextual fear conditioning has been useful in discovering gene targets, gene expression after spatial memory tasks has received less attention. In this study, we used the object-location memory task and studied gene expression at two time points after learning in a high-throughput manner using a microfluidic qPCR approach. We found that expression of the classic immediate-early genes changes after object-location training in a fashion similar to that observed after contextual fear conditioning. However, the temporal dynamics of gene expression are different between the two tasks, with object-location memory producing gene expression changes that last at least 2 hours. Our findings indicate that different training paradigms may give rise to distinct temporal dynamics of gene expression after learning.

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Keywords

transcription; spatial memory; fear conditioning; object-location memory; hippocampus

1. Introduction

Long-term memory is critical to our lives, yet the molecular mechanisms that create and stabilize memories are still poorly understood. The hippocampus, which encodes contextual information, has been heavily studied in an effort to better understand these mechanisms. Transcription is required to convert labile short-term memories into stable long-term memories during the period of memory consolidation (Agranoff et al, 1967; Igaz et al, 2002). The expression of many genes is regulated within the first hour after learning in the hippocampus (Hawk et al, 2012; Keeley et al, 2006; Lemberger et al, 2008; Levenson et al, 2004a; Lonergan et al, 2010; Ramamoorthi et al, 2011). Epigenetic mechanisms, such as histone acetylation, can modulate this transcription to enhance or dampen long-term memory formation (Alarcon et al, 2004; Guan et al, 2009; Kozus et al, 2004; Levenson et al, 2004b; McQuown et al, 2011; Vecsey et al, 2007; Wood et al, 2006; Wood et al, 2005).

Most research into transcriptional regulation in the hippocampus has used contextual fear conditioning as the paradigm to test learning and memory (Barnes et al, 2012; Keeley et al, 2006; Levenson et al, 2004a; Mei et al, 2005). This is primarily because contextual fear conditioning produces a robust memory that has a well-defined time of acquisition due to the requirement of only a single training session (Abel & Lattal, 2001). Although this task has proven useful for dissecting the phases of memory and mapping the transcriptional landscape after learning, it also introduces a footshock that can be stressful to the animal. It is therefore important to study gene expression in other memory tasks that are more similar to the learning events that occur in daily life.

Spatial learning requires the hippocampus and can be measured using the Morris water maze, Barnes maze, or object-location memory (OLM) tasks that do not require a footshock. These spatial tasks are also known to regulate transcription in the hippocampus, including many of the same genes and processes required for contextual fear memory (Bousiges et al, 2010; Cavallaro et al, 2002; Florian et al, 2006; Fordyce et al, 1994; Haettig et al, 2011; Hawk et al, 2011; Klur et al, 2009; McNulty et al, 2012; Pittenger et al, 2002; Vogel-Ciernia et al, 2013). There is evidence that contextual and spatial learning in the hippocampus can utilize different molecular pathways (Mizuno & Giese, 2005), so gene expression may also differ after these two tasks. Like contextual fear memory, OLM is a hippocampus-dependent task (Oliveira et al, 2010). However, the targets and temporal resolution of the gene expression changes after OLM have not been thoroughly studied. The goal of this study was to investigate the transcriptional profile that occurs within the first transcriptional wave after OLM learning (Bourtchouladze et al, 1998; Igaz et al, 2002) and compare this transcriptional profile to that of contextual fear conditioning. Gene expression changes within this window after fear conditioning are typically highest 30 minutes after training and return to baseline by 2 hours (Hawk et al, 2012; Keeley et al, 2006; Peixoto et al, 2013). Using a Fluidigm HD microfluidic high-throughput qPCR system, we examined expression

of 96 different candidate genes at both 30 minutes and 2 hours after OLM training in a single experiment. We found that the most commonly studied genes after fear conditioning show a similar profile after OLM. However, OLM produces long-lasting expression changes in a number of genes that are not observed after fear conditioning.

2. Methods

2.1. Subjects

Forty-two C57BL/6J mice were maintained under standard conditions with food and water available *ad libitum*. Adult male mice 3 months of age were kept on a 12-hr light/12-hr dark cycle with lights on at 7AM. All behavioral and biochemical experiments were performed during the light cycle with training starting at approximately 7AM (ZT0). All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

2.2. Behavior

Object-location memory (OLM) was carried out as previously described (Hawk et al, 2011; Oliveira et al, 2010). Briefly, naïve three month old male C57Bl/6J mice were singly housed for a week and handled for 2 min/day for five consecutive days prior to tissue collection. One animal per behavioral group was trained and dissected each day for 10 total days to allow all animals to be dissected at the same circadian time. Exploration was normal in all mice used in this experiment (data not shown). One animal per training session was tested in a 24hr retrieval test the following day to ensure the training proceeded correctly. Half of the handled animals received OLM training, and half of the animals were left undisturbed on training day and were sacrificed at the same circadian time points as trained animals. On the day of training, OLM mice were given a single block of four 6 min trials with an inter-trial interval of 3 min. The animals were habituated to an empty arena with a black and white striped spatial cue on one wall in the first trial, followed by three trials of object exposure. Each mouse was exposed to three distinct objects: a rectangular metal tower, a glass bottle, and a white plastic cylinder that were arranged in a V-shaped spatial pattern in the arena. Objects were positioned in the arena with at least two inches of spacing around each object to allow free exploration of all objects. During the ITI, animals were gently removed from the arenas, and the arenas and objects were cleaned with 70% ethanol. Objects were not moved during the ITI. Immediately following the final trial, animals were gently placed in their home cage, and returned to the colony room until tissue collection.

Fear conditioning was performed as previously described (Hawk et al, 2012; Vecsey et al, 2007) with handling for 3 days prior to conditioning. Briefly, the conditioning protocol entailed a single 2-sec, 1.5mA footshock terminating at 2.5 minutes after placement of the mouse in the novel chamber. Mice were left in the chamber for an additional 30 seconds and then returned to their homecage.

2.3 RNA isolation

Hippocampi were dissected 30 minutes and 2 hours after the last training session into RNAlater (Qiagen, Valencia, CA) and frozen on dry ice. Tissue was homogenized using a

TissueLyser system and RNA was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's instructions.

2.4 cDNA synthesis and high-throughput qPCR

RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and 1 μ g RNA was used in each RETROscript (Ambion, Austin, TX) cDNA synthesis reaction with random decamers, 10x RT Buffer and no heat denaturation according to the manufacturer's protocol. Concentrated cDNA was used in a specific target reaction following the manufacturer's recommendations (Fluidigm Corp. South San Francisco, CA). Briefly, Taqman assays for all 96 probes were pooled to a concentration of 0.2X (1:100) and 1.25 μ l of the pooled assay mix was combined with 2.5 μ l 2X Taqman Preamp Master Mix (Life technologies, Carlsbad, CA) and 1.25 μ l cDNA. Taqman probe IDs can be found in Table S1. The preamplification reaction was cycled using the following protocol in a 7500 Fast Real-Time PCR system: 10 min at 95C, then 14 cycles of 95C for 15s followed by 60C for 4min. Preamplified samples were diluted 1:5 using 1X TE. Samples were then delivered to the Molecular Profiling Core at the University of Pennsylvania, where they were run on a 96.96 Dynamic Array IFC on the Biomark HD machine (Fluidigm Corp).

For validation and the 120 minute fear conditioning experiment, cDNA reactions were diluted to 2 ng/ μ l in water, and real-time RT-PCR reactions were prepared in 384-well optical reaction plates with optical adhesive covers (Life technologies). Each reaction was composed of 2.25 μ l cDNA (2 ng/ μ l), 2.5 μ l 2x Taqman Fast Universal Master Mix (Life Technologies), and 0.25 μ l of Taqman probe. Reactions were performed in triplicate on the Viia7 Real-Time PCR system (Life Technologies, Carlsbad, CA).

2.5 Data analysis

High-throughput qPCR was analyzed using the Fluidigm Real Time PCR Analysis program and Microsoft Excel. Genes with at least one sample having an average Ct \geq 20 were discarded as being non-expressed or failed reactions. This included Dnmt3b, Erbb2, Esrrg, Fosb, Hdac1, Hdac4, Jun, Nr6a1, Pparg, and Trdmt1, which brought the total number of genes tested to 86. Relative quantification of gene expression between groups was performed using the Δ Ct method as described previously (Vecsey et al, 2007). The difference between each Ct and the average Ct for that gene was subtracted from the average of three housekeeper genes treated in the same manner. A p-value of 0.01 was used for significance to control for the number of t-tests performed. This p-value cutoff was chosen because we selected genes for analysis that we expected to change, and thus Bonferroni correction is too strict. This 1% chance of a type I error corresponds to one false positive per 100 t-tests. Because 86 t-tests were performed, this p-value would suggest less than 1 false positive in the data, limiting the amount of type I errors introduced by multiple testing.

3. Results

3.1 Immediate Early Genes Are Regulated 30 minutes after OLM training

We chose sixteen representative genes that have been studied 30 minutes after fear conditioning to examine expression profiles 30 minutes after OLM training. The genes were chosen for well-studied expression changes (*Arc*, *Bdnf4*, *Egr1*, *Fos*, *Homer1*), genes our lab has previously studied [*Fosl2* (Hawk et al, 2012), *Gadd45* family (Leach et al, 2012)], or from microarray data (*Btg2*, *Cpeb3*, *Histh2hab*, *Sik1*, *Sox18*, *Tob1*, *Tob2* (Peixoto et al, 2013)). cDNA samples underwent specific target amplification and were run on a 96.96 Fluidigm Biomark HD plate in triplicate (96 genes, 32 samples). The full list of Taqman assays is available in Table S1. Ten genes were excluded due to too low expression or a failed reaction, bringing the total number of genes tested to 86 (See Methods for genes). In all cases, immediate early gene (IEG) expression after OLM mirrored expression after fear conditioning (Figure 1). Previously studied genes including *Arc*, *Bdnf4*, *Egr1*, *Fos* and *Homer1* were upregulated as anticipated (*Arc* 272% $p=4.7\times 10^{-8}$; *Bdnf4* 53% $p=3.3\times 10^{-7}$; *Egr1* 225% $p=4.4\times 10^{-8}$; *Fos* 410% $p=1.2\times 10^{-10}$; *Homer1* 31% $p=1.4\times 10^{-4}$) (Keeley et al, 2006; Lonergan et al, 2010; Mahan et al, 2012; Mizuno et al, 2012). The probe against *Homer1* recognizes both *Homer1a* and *Homer1c*, but research from our lab and others suggests that this effect is primarily due to *Homer1a* (Mahan et al, 2012). Further investigation is required to investigate specific *Homer1* isoforms regulated by OLM. Genes that our lab discovered to be regulated after contextual fear conditioning using microarrays (Peixoto et al, 2013), including *Btg2* (27% $p=8.9\times 10^{-6}$), *Hist2h2ab* (-26% $p=8.8\times 10^{-4}$), *Sik1* (70% $p=1.3\times 10^{-5}$), *Sox18* (-21% $p=0.002$), and *Tob2* (30% $p=0.004$) showed similar changes after OLM. The genes *Gadd45b* and *Gadd45g* showed increased expression (32% $p=0.004$, 32% $p=0.001$) while *Gadd45a* did not ($p=0.20$), as has been reported previously by our lab and others (Leach et al, 2012; Sultan et al, 2012). This observation suggests that the most commonly studied genes after contextual fear conditioning are similarly regulated after spatial behavioral tasks such as object-location memory.

3.2 Nuclear Hormone Receptors Display a Limited Response to OLM

A subset of nuclear hormone receptors are known to be regulated 30 minutes after fear conditioning, including the *Nr4a* family of orphan nuclear receptors (Hawk et al, 2012). We tested all 37 nuclear hormone receptors that are expressed in the hippocampus for changes after OLM training (Figure 2). The *Nr4a* family of nuclear receptors (*Nr4a1*, *Nr4a2*, *Nr4a3*), which are known to be necessary for long-term fear memory (Hawk et al, 2012; McNulty et al, 2012; McQuown et al, 2011), all displayed increased expression at 30 minutes after OLM. Rev-ErbA (*NR1D1*), COUP-TFII (*NR2F2*), and retinoid X receptor gamma (*Rxrg*) all showed decreased expression at 30 minutes. No other nuclear receptors were observed to respond to spatial learning at this time point or at 120 minutes after OLM (data not shown). This contrasts with the large number of nuclear receptors that our lab observed to change after fear conditioning training in our previous study (Hawk et al, 2012), which included increased expression of 13 nuclear receptor genes between 30 and 120 minutes after training. These results may indicate transcriptional regulation of this class of genes depends on the training paradigm.

3.3 Regulators of Transcription Show Limited Changes in Response to OLM

Histone acetylation is known to be a crucial regulator of transcription during memory consolidation (Aларcon et al, 2004; Barrett et al, 2011; Haettig et al, 2011; Korzus et al, 2004; Levenson et al, 2004b; McQuown et al, 2011; Vecsey et al, 2007; Wood et al, 2006; Wood et al, 2005). To test whether expression levels of histone acetylation modifying enzymes are regulated by OLM, we tested all histone deacetylases (HDACs, Figure 3A) and 16 histone acetyltransferases (HATs, Figure 3B) representing each class of enzyme, including the HATs CBP and p300 that have been shown to be essential for memory formation. The probes against *Hdac1* and *Hdac4* did not amplify and were discarded. None of the HATs tested showed a gene expression change, in contrast to previous reports showing changes in expression of CBP, p300 and PCAF after the Morris Water Maze (Bousiges et al, 2010). However, *Hdac7* displayed reduced expression after OLM. HDAC7 is a class IIa HDAC that has not been previously linked to memory formation. This may suggest a novel role for HDAC7 in hippocampus-dependent memory formation. In addition to the regulators of histone acetylation, we chose ten genes that are known to regulate transcription in other ways. None of these genes showed any changes in transcription at 30 minutes after OLM training (Figure 4).

3.4 OLM Induces Longer Lasting Gene Expression Changes than Fear Conditioning

In addition to the 30 minute timepoint that has shown such robust changes after fear conditioning, we also tested hippocampal samples taken 2 hours after OLM training to investigate the persistence of these transcriptional changes. Interestingly, a number of genes that are upregulated at 30 minutes remain elevated 2 hours after OLM training. This includes highly induced genes that appear to be slowly returning to baseline, such as *Egr1* and *Fos*, but also genes that maintain a similar level of induction as observed at 30 minutes such as *Bdnf4*, *Fosl2*, *Homer1*, *Nr4a2* and *Nr4a3* (Figure 5A). *Sin3a* was not changed at 30 minutes, but shows a selective change at 2 hours. The gene expression profiles at 30 minutes and 2 hours for *Arc*, *Egr1*, *Fos*, *Nr4a1*, and *Nr4a2* were confirmed by standard 384-well qPCR (data not shown). To test whether these same genes show transcriptional changes after fear conditioning, we prepared cDNA from samples that were collected 2 hours after fear conditioning. None of the genes determined to change 2 hours after OLM showed a significant change 2 hours after fear conditioning (Figure 5B), indicating a long-lasting gene expression response specific to OLM.

4. Discussion

In this study, we investigated the transcriptional changes that occur in response to OLM training using powerful high-throughput qPCR technology and compared these changes to fear conditioning. In a single run, we were able to study 96 different genes in 2 different time points after OLM training with n=8 mice per group using microfluidic high-throughput qPCR. This type of throughput, flexibility, and consistency is not possible with any other qPCR technology. In addition to requiring more pipetting steps, standard qPCR would have required the same housekeepers to be run on each individual plate and limited the number of targets that could be tested. Using a high-throughput approach allowed us to reliably

determine that gene expression changes after OLM last longer than similar expression changes after contextual fear.

Our study discovered that commonly studied IEGs, such as *Fos* and *Arc*, show similar expression differences after fear conditioning and after OLM, indicating overlap between contextual and spatial learning. In a previous study from our lab (Hawk et al, 2012), we found that a number of nuclear receptors exhibit increased expression after contextual fear conditioning. Our current findings suggest a more limited regulation of this class of genes after OLM. It is unclear whether the wider regulation after fear conditioning is in response to the footshock or whether the timecourse of expression after OLM is different. As seen after fear conditioning, all 3 members of the *Nr4a* family of orphan nuclear receptors were upregulated after OLM. However, while *Nr4a1* returned to baseline by 2 hours, *Nr4a2* and *Nr4a3* did not, suggesting that different processes may regulate *Nr4a1* than the other two family members. Future studies will aim to determine how expression increases of *Nr4a2* and *Nr4a3* are maintained after OLM training.

It is interesting to note that *Hdac7* and *Sin3a* are regulated by OLM while HATs are not. This may suggest that relieving the negative repression of histone acetylation is a crucial step for long-term memory formation. Although class I HDACs have been heavily implicated in learning and memory (Bahari-Javan et al, 2012; Guan et al, 2009; Hawk et al, 2011; McQuown et al, 2011), class IIa HDACs have received less attention. A study by Agis-Balboa et al. demonstrated that loss of the class IIa member HDAC5 impairs spatial memory (Agis-Balboa et al, 2013), but those experiments used a complete knockout mouse line that has the potential for developmental or extrahippocampal effects. Our study is the first to observe changes in *Hdac7* in response to learning in the hippocampus.

The most intriguing finding of this study was the long-lasting regulation of gene expression 2 hours after OLM, something that is not seen after fear conditioning. It might be expected that the fear of a footshock would produce a stronger transcriptional response in the hippocampus than would the spatial rearrangement of objects. There are a number of potential causes for this disparity, although the most likely explanation is that the multiple training sessions required for OLM induce a stronger response than the single shock training used by our lab for fear conditioning. It would be interesting to test whether a multiple shock fear conditioning protocol induces longer lasting gene expression changes. Also, there could be an association between the novel context and the novel objects formed during OLM training that is not present in fear conditioning. Testing mice in the context only, introducing novel objects, or altering the number of training trials could determine whether these changes are sufficient to elicit gene expression changes. Further, different molecular mechanisms may regulate contextual and spatial learning (Mizuno & Giese, 2005). Future studies can test for changes at the protein level, although mRNA and protein levels generally agree after learning (Stanciu et al, 2001; Steward et al, 1998). Additional investigation into later time points after OLM training will be required to see if gene expression changes that occur well after fear conditioning (Mizuno et al, 2012) also exist after OLM. It is interesting that not all genes with increased expression at the 30 minute timepoint remain elevated for 2 hours after OLM. Future studies will determine whether specific epigenetic modifications regulate this longer term maintenance of gene expression at particular genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Microfluidic technology allows for large-scale qPCR experiments
- Object-location memory increases IEG expression similarly to fear conditioning
- Object-location memory induces gene expression changes that last at least 2 hours

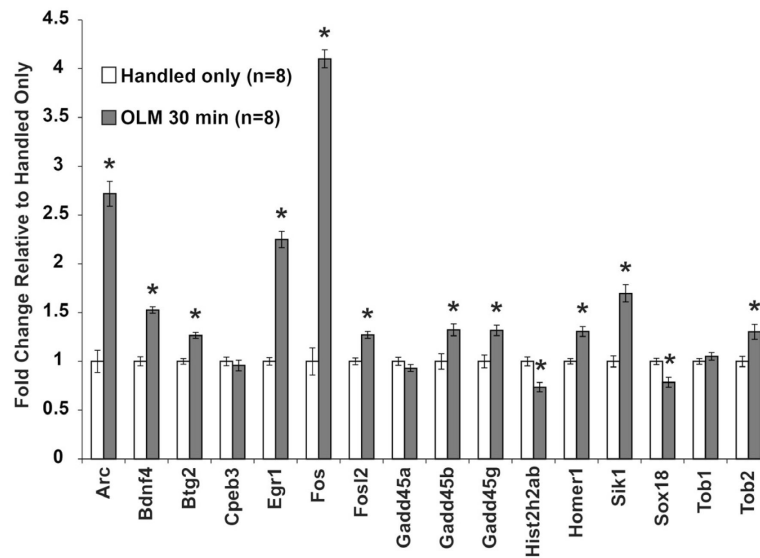


Figure 1. Classic IEGs Show Expected Expression Changes after OLM Training

Sixteen genes that are known to be induced 30 minutes after contextual fear conditioning were studied 30 minutes after OLM training. Each gene tested displayed the expression change that would be expected after contextual fear conditioning indicating these genes may represent a common transcriptional response to learning. All error bars denote s.e.m. and * indicates $p < 0.01$.

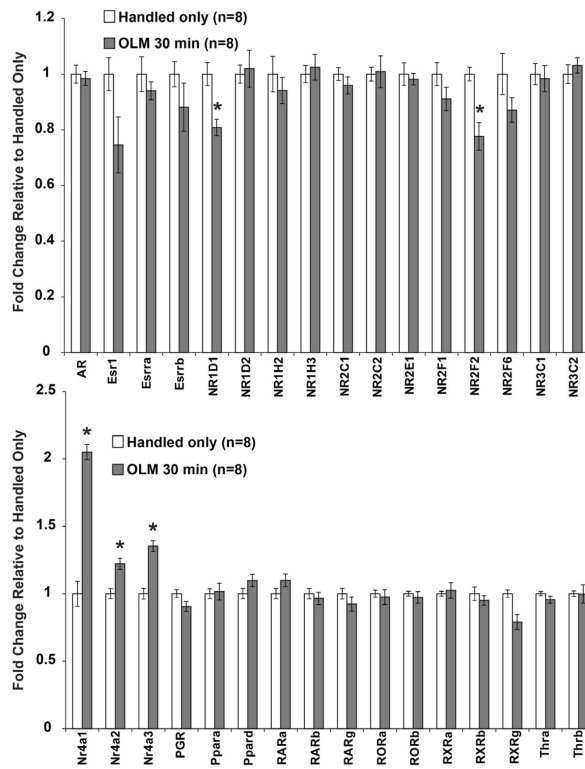


Figure 2. Limited Expression Changes of Nuclear Receptors after OLM training

Because of the known involvement of the *Nr4a* nuclear receptor family in memory, we tested expression of all nuclear receptors expressed in the hippocampus 30 minutes after OLM training. The *Nr4a* family displayed increased expression after OLM, while *NR1D1*, *NR2F2*, and *RXRg* had reduced expression. All error bars denote s.e.m. and * indicates $p < 0.01$.

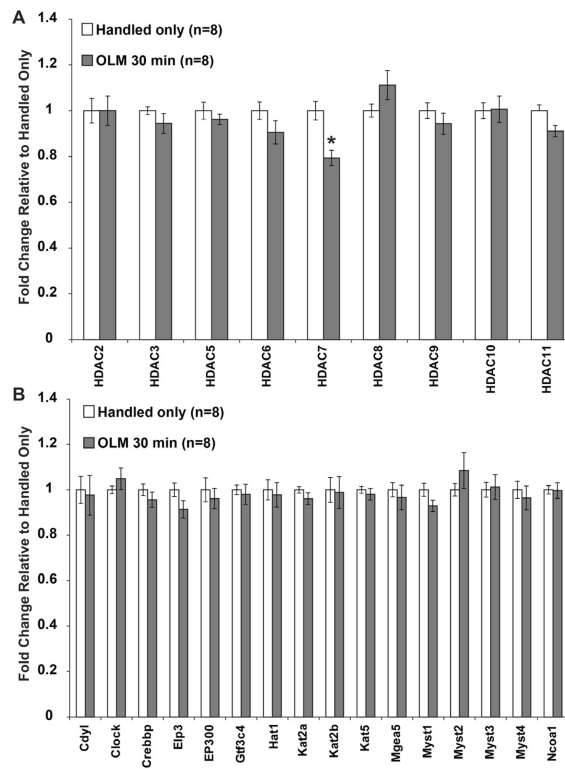


Figure 3. Modifiers of Histone Acetylation Display Limited Regulation after OLM Training
 Histone modifying enzymes were tested for expression changes 30 minutes after OLM training. (A) *Hdac7*, a class IIa HDAC, was the only family member found to change expression after OLM. (B) No HATs were observed to change expression after OLM. All error bars denote s.e.m. and * indicates $p < 0.01$.

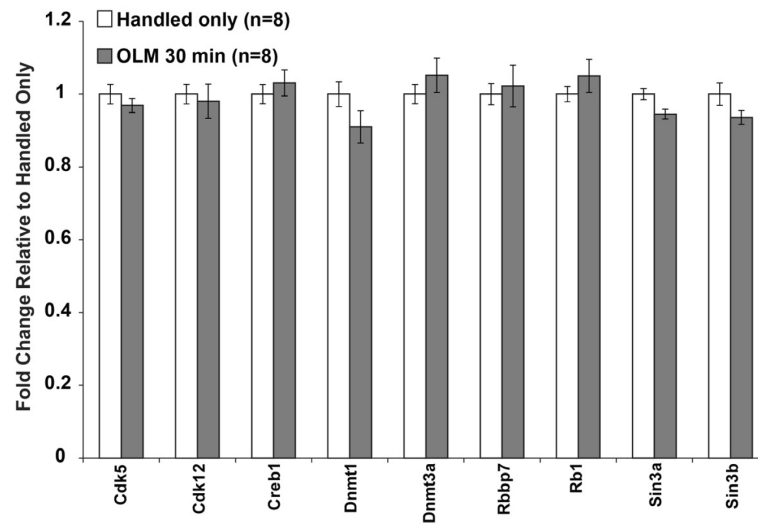


Figure 4. No Changes in Other Transcriptional Regulators after OLM Training

Other genes that can regulate gene expression, including DNMTs, were tested 30 minutes after OLM training. No differences in any gene were observed. All error bars denote s.e.m. and * indicates $p < 0.01$.

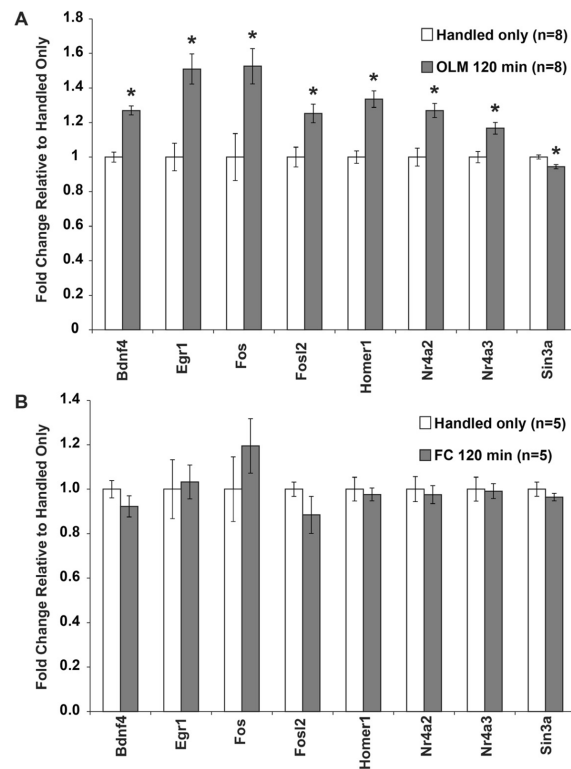


Figure 5. OLM Training Induces Long-Lasting Changes in Gene Expression Not Seen after Fear Conditioning

(A) Every gene was also tested 2 hours after OLM training to observe the maintenance of transcription. Genes shown in this figure are those that were changed at 2 hours after OLM, all other genes were unchanged. *Sin3a* was the only gene uniquely regulated at the 2 hour time point. (B) These same genes do not show gene expression changes 2 hours after contextual fear conditioning. All error bars denote s.e.m. and * indicates $p < 0.01$.