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NR4A Nuclear Receptors Support Memory Enhancement by Histone Deacetylase Inhibitors

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NR4A Nuclear Receptors Support Memory Enhancement by Histone Deacetylase Inhibitors

Abstract

The formation of a long-lasting memory requires a transcription-dependent consolidation period that converts a short-term memory into a long-term memory. Nuclear receptors compose a class of transcription factors that regulate diverse biological processes, and several nuclear receptors have been implicated in memory formation. Here, we examined the potential contribution of nuclear receptors to memory consolidation by measuring the expression of all 49 murine nuclear receptors after learning. We identified 13 nuclear receptors with increased expression after learning, including all 3 members of the *Nr4a* subfamily. These CREB-regulated *Nr4a* genes encode ligand-independent “orphan” nuclear receptors. We found that blocking NR4A activity in memory-supporting brain regions impaired long-term memory but did not impact short-term memory in mice. Further, expression of *Nr4a* genes increased following the memory-enhancing effects of histone deacetylase (HDAC) inhibitors. Blocking NR4A signaling interfered with the ability of HDAC inhibitors to enhance memory. These results demonstrate that the *Nr4a* gene family contributes to memory formation and is a promising target for improving cognitive function.

Disciplines

Amino Acids, Peptides, and Proteins | Medical Neurobiology | Nervous System | Neuroscience and Neurobiology | Neurosciences

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NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors

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The formation of a long-lasting memory requires a transcription-dependent consolidation period that converts a short-term memory into a long-term memory. Nuclear receptors compose a class of transcription factors that regulate diverse biological processes, and several nuclear receptors have been implicated in memory formation. Here, we examined the potential contribution of nuclear receptors to memory consolidation by measuring the expression of all 49 murine nuclear receptors after learning. We identified 13 nuclear receptors with increased expression after learning, including all 3 members of the *Nr4a* subfamily. These CREB-regulated *Nr4a* genes encode ligand-independent “orphan” nuclear receptors. We found that blocking NR4A activity in memory-supporting brain regions impaired long-term memory but did not impact short-term memory in mice. Further, expression of *Nr4a* genes increased following the memory-enhancing effects of histone deacetylase (HDAC) inhibitors. Blocking NR4A signaling interfered with the ability of HDAC inhibitors to enhance memory. These results demonstrate that the *Nr4a* gene family contributes to memory formation and is a promising target for improving cognitive function.

Introduction

Memories are initially stored in a fragile form that can be disrupted by new information, but in the hours following learning a transcription-dependent process known as memory consolidation converts these short-term memories into stable long-term memories. The cellular mechanisms governing memory consolidation have been the subject of intense study over the past 30 years. The molecular underpinnings of memory consolidation have been most thoroughly studied in a region of the brain known as the hippocampus during spatial and contextual memory formation (1). Hippocampus-dependent memory formation requires 2 waves of protein synthesis (2), cAMP-dependent kinase (PKA) activity (2), and de novo transcription in the hippocampus (3) in the hours following learning.

Nuclear receptors (NRs) compose the largest class of transcription factors found in metazoans (4). Generally, NRs are regulated by lipophilic ligands, allowing rapid, ligand-dependent control of various developmental and metabolic processes. This family includes receptors for fat-soluble vitamins, endocrine hormones, thyroid hormones, fatty acids, bile acids, oxysterols, and dietary xenobiotic lipids. Additionally, “orphan” NRs either have no ligand or a ligand that has yet to be identified. Several NRs have been implicated in the formation of memory. For instance, agonists for glucocorticoid receptors, estrogen receptors (ERs), PPARs, and retinoic acid receptors (RARs) can improve long-term memory formation under certain conditions (5–8). Additionally, mice with mutations in the *Erb* (9), *Rarb* (10), or the orphan NR *Nr4a2* have deficits in long-term memory (11).

Despite the importance of NRs to diverse physiological processes and data supporting a role of select NRs in memory formation,

a systematic analysis of NR expression after learning has not been previously performed. Therefore, we surveyed the expression of all 49 NR genes after learning in the single-trial contextual fear-conditioning task. This training protocol produces a robust memory that requires the hippocampus, a site of increased gene expression after learning (12). We examined time points spanning the entire 24-hour period after learning and found that 13 NRs have increased hippocampal expression in the first 2 hours after training. Among these 13 learning-induced NRs were all 3 members of the *Nr4a* orphan NR family.

Interestingly, *Nr4a* family gene expression is activated by many of the same signaling cascades that are required for long-term memory formation, including cAMP, PKA, and cAMP-response element-binding protein (CREB) (reviewed in ref. 1). Further, a class of drugs that improves long-term memory formation through inhibition of histone deacetylases (HDACs) increases the expression of *Nr4a* genes (13). Therefore, we used a dominant-negative strategy to ascertain whether NR4A signaling contributes to long-term memory formation and the enhancement in memory caused by HDAC inhibitors. We found that transgenic expression of a dominant-negative form of NR4A in forebrain neurons impairs long-term contextual memory consolidation and blocks memory enhancement by intrahippocampal infusion of HDAC inhibitors after training. Further, we identify *Bdnf* and *Fosl2* as targets of NR4A signaling that are also enhanced by HDAC inhibitor treatment. These results demonstrate a role for NR4A signaling in long-term memory formation and the enhancement in memory by HDAC inhibitors.

Results

NR gene expression in the hippocampus is regulated by contextual learning. To address whether NR gene expression might be associated with memory consolidation, we examined hippocampal gene expres-

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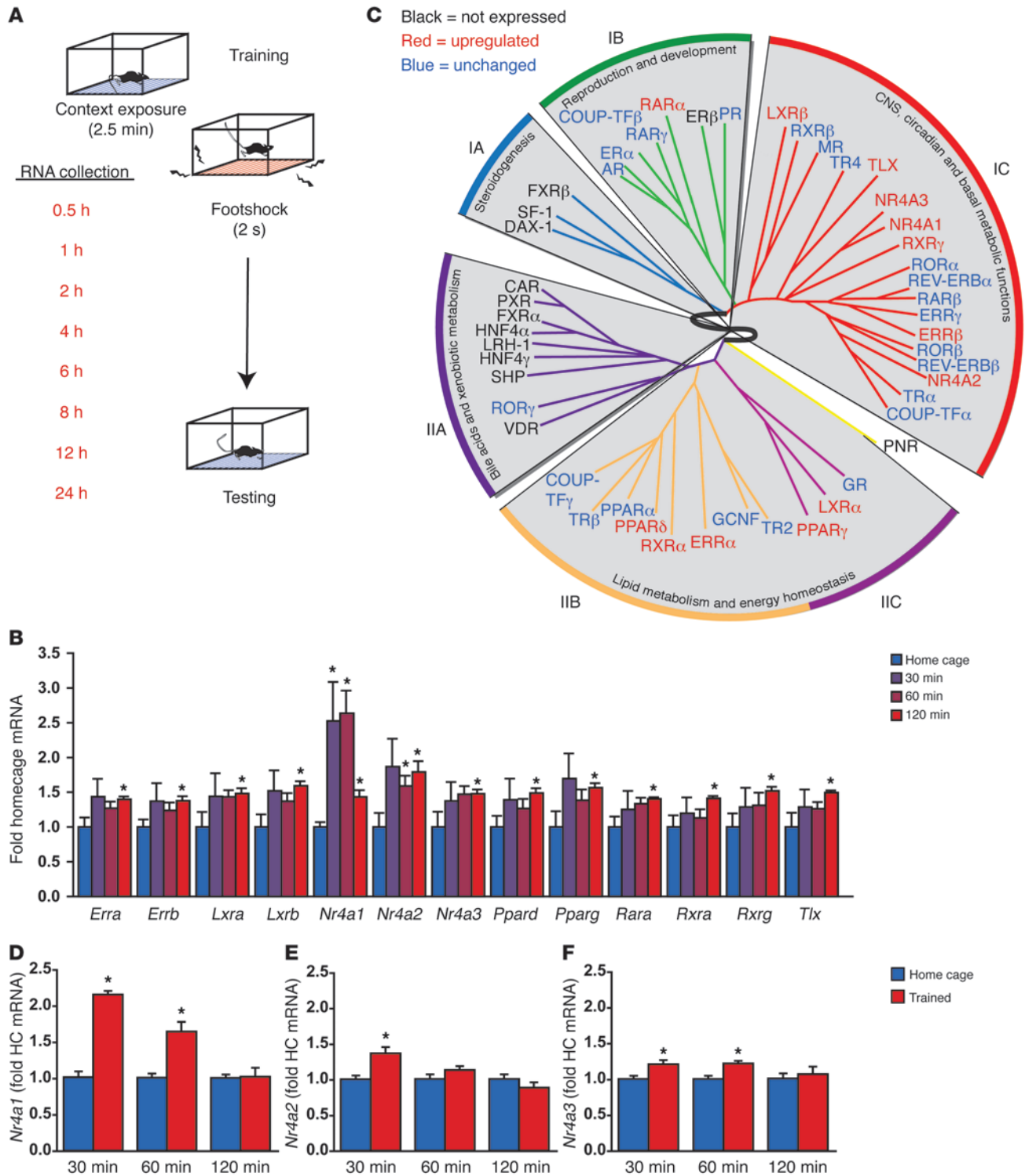




Figure 1

The formation of contextual fear memories induces expression of NR genes in the hippocampus. **(A)** Contextual fear conditioning produces a long-lasting memory for the training context and the association of this context with a mild foot-shock. RNA was collected from whole hippocampi at multiple time points after training to survey the impact of training on NR gene expression using a standard $\Delta\Delta\text{CT}$ approach. **(B)** High-throughput qPCR data are illustrated for the 13 NR genes with statistically significant changes in gene expression during the first 2 hours after training, the window in which the majority of changes were observed. **(C)** The data from this screen indicate that 13 NRs have increased expression in the hippocampus within the first 2 hours after training (red), whereas 13 NRs are not appreciably expressed in the hippocampus (black). The remaining 23 NRs (blue) show no evidence of altered hippocampal expression in the first 2 hours after training. Expression changes are illustrated within clusters defined by anatomical expression profiling (IA, IB, IC, IIA, IIB, IIC) in a diagram modified with permission from *Cell*; ref. 17. **(D)** *Nr4a1* expression is potently induced in the first hour after learning ($P < 0.001$). **(E)** *Nr4a2* expression increases after fear conditioning ($P = 0.033$). **(F)** *Nr4a3* expression increases after fear conditioning ($P = 0.004$). HC, home cage. Error bars represent SEM. * $P < 0.05$. See also Supplemental Figure 1.

sion after contextual fear conditioning, a form of hippocampus-dependent memory (14). We chose this task because the anatomical circuitry and molecular signaling cascades underlying this form of memory are well established. Additionally, the timings of these molecular signaling events are directly measurable relative to a single training episode. Contextual fear conditioning is associated with 2 waves of CREB phosphorylation after training (15), and long-term contextual fear memory is sensitive to inhibitors of translation or PKA during 2 time windows that coincide with these 2 peaks of CREB phosphorylation (2). The first of these windows occurs within the first hour after learning, and the second occurs between the third and sixth hour after learning (2, 15). Newly expressed genes, such as *Bdnf*, appear to contribute to memory formation even as late as 12 hours after acquisition of fear memories (16). For these reasons, we analyzed gene expression in the hippocampus at multiple time points after learning (0.5, 1, 2, 4, 6, 8, 12, and 24 hours) in comparison to circadian controls (Figure 1A).

Out of the 49 NRs, 13 were not expressed at appreciable levels in the hippocampus (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI64145DS1). These 13 genes cluster into the steroidogenesis and bile acid and xenobiotic metabolism functional categories (Figure 1C) identified previously by anatomical profiling of NRs (17). Of those genes expressed in the hippocampus, very few showed any evidence of altered gene expression in the late time points, more than 2 hours after learning (Supplemental Figure 1B). For this reason, we have focused on changes in the first 2 hours after training (Figure 1C).

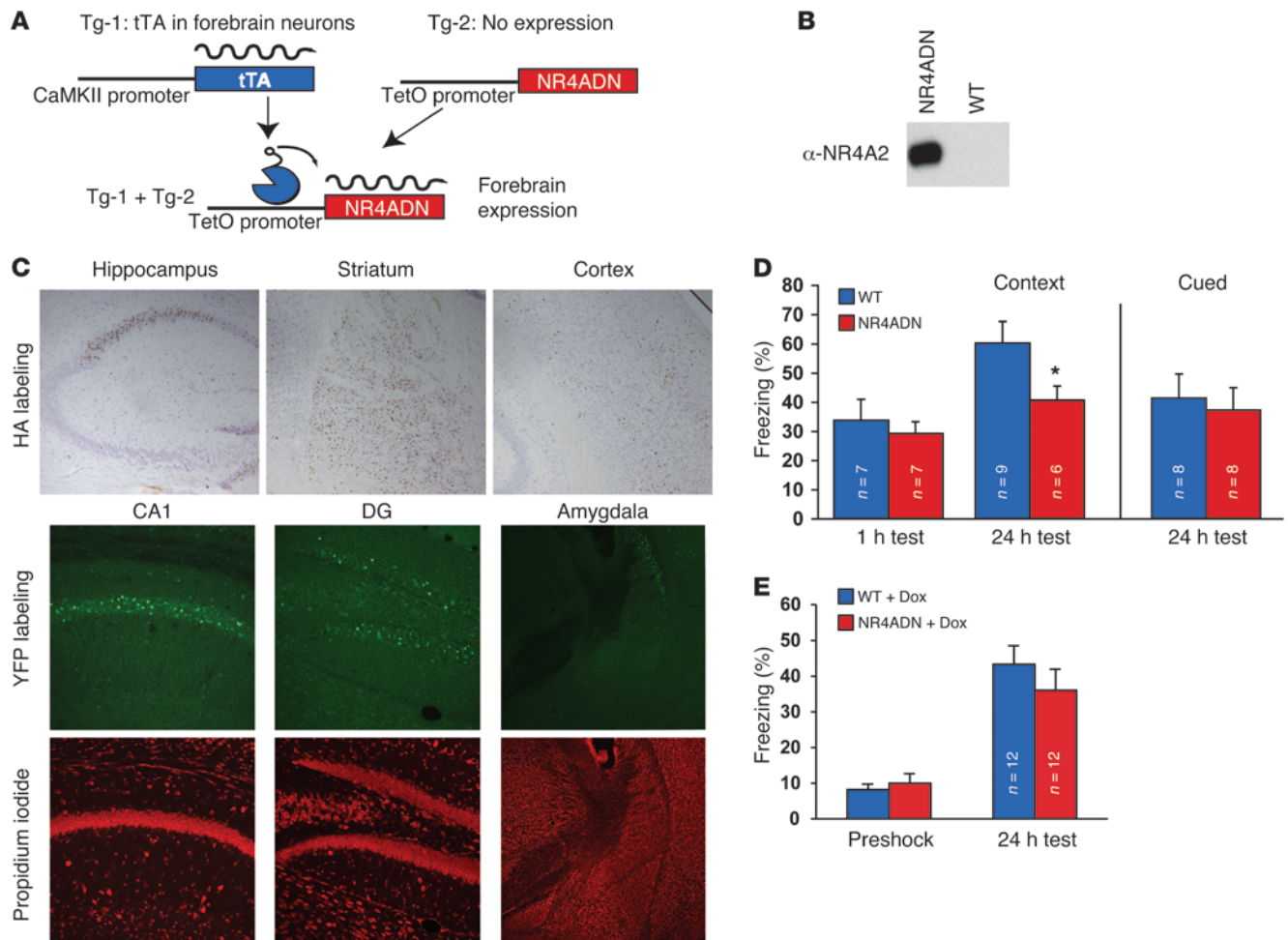
Another 13 NRs showed increased expression in the first 2 hours after training (Figure 1B, red in Figure 1C). These 13 genes encode 6 orphan NRs (*Tlx*, also known as *Nr2e1*; *NGFI-B*, also known as *Nr4a1*; *Nurr1*, also known as *Nr4a2*; *Nor1*, also known as *Nr4a3*; *Erra*, also known as *Nr3b1*; *Errb*, also known as *Nr3b2*) and 7 receptors with known ligands (*Lxra*, also known as *Nr1h3*; *LXRβ*, also known as *Nr1h2*; *Ppard*, also known as *Nr1c2*; *Pparg*, also known as *Nr1c3*; *Rara*, also known as *Nr1b1*; *Rxra*, also known as *Nr2b1*; *Rxrg*, also known as *Nr2b3*). Another 23 of the NRs were expressed in the hippocampus, but mRNA levels of these genes were not altered in the 2-hour window after training (blue in Figure 1C).

NR4A subfamily gene expression is regulated by contextual learning. NRs can be grouped into 6 functionally – and evolutionarily – related subfamilies (NR1–NR6) and another “catch-all” subfamily (NR0) (18). Four of these seven subfamilies are represented with the group of training-induced genes found and shown in Figure 1, B and C; however, only the NR4 subfamily shows evidence for the entire family being induced by learning. The *Nr4* gene family encodes 3 NR4A NRs: *NR4A1* (also known *NGFI-B*, *NUR77*, and *TR3*), *NR4A2* (also known as *NURR1*, *HZF-3*, and *RNR1*), and *NR4A3* (also known as *NOR1*, *MINOR*, and *TEC*). Consistent with our results, in situ analysis has previously shown that *Nr4a1* expression increases in hippocampal area CA1 (19) and that *Nr4a2* expression increases in hippocampal areas CA1 and CA3 (20) following hippocampus-dependent learning. Additionally, *Nr4a2* heterozygous null mice have impaired hippocampus-dependent passive avoidance memory (11), and *Nr4a2* knockdown using antisense oligodeoxynucleotide injection into the hippocampus impairs long-term memory in a spatial discrimination task (21). Further, improved long-term spatial memory performance observed by deletion of HDAC3 in the hippocampus is attenuated by treatment with siRNA targeting *Nr4a2* (22). Therefore, we chose to pursue the *Nr4a* gene family for follow-up studies by first confirming that expression of each of the 3 *Nr4a* genes increases after fear conditioning using low-throughput qPCR methods. Contextual fear conditioning had a significant overall effect on gene expression for each of the 3 *Nr4a* genes (*Nr4a1*, $P < 0.001$, Figure 1D; *Nr4a2*, $P = 0.033$ Figure 1E; *Nr4a3*, $P = 0.004$, Figure 1F). We found that *Nr4a1* expression increases substantially at 30 minutes after contextual fear conditioning ($P = 0.009$), with transcript levels remaining elevated at 60 minutes after training ($P = 0.009$) but returning to baseline by 120 minutes after training. A similar pattern of expression was observed for *Nr4a3* (30 minutes, $P = 0.028$; 60 minutes, $P = 0.016$). *Nr4a2* expression was significantly elevated at 30 minutes after training ($P = 0.009$). Thus, *Nr4a* family gene expression increases within the hippocampus after training in a hippocampus-dependent task.

Generation of a NR4A dominant-negative transgenic mouse line. Our data demonstrate that learning induces de novo gene expression for all 3 *Nr4a* family genes during a window in which new gene synthesis is required for long-term memory formation (1). Because NR4A proteins are ligand-independent NRs (23), the level of gene expression is a major factor determining NR4A activity. In the hippocampus, *Nr4a1* and *Nr4a2* are 2 out of only 19 immediate early genes for which induction was blocked in CREB conditional mutant mice after seizure activity (24). Thus, *Nr4a* expression may be an important part of a CREB-initiated cascade of gene expression that contributes to the formation of long-term memory. This hypothesis is supported by existing data suggesting a role for *Nr4a2* in long-term memory formation (11, 21). In other biological contexts, *Nr4a* family members have been observed to have redundant functions (25, 26). In these situations, a truncated version of NR4A1 that acts as a dominant-negative protein to silence all 3 family members has been an invaluable tool in deciphering the physiological roles of NR4A signaling (25, 27). Further, the involvement of NR4A signaling in distinct processes in different cell types (28, 29) and in different anatomical regions (29) calls for a cell-type and regionally restricted approach to examine the role of NR4A signaling in memory formation. For these reasons, we adapted the NR4A dominant-negative approach to allow us to block NR4A signaling in hippocampal neurons. The dominant-negative form of



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**Figure 2**

Blocking function of NR4A family NRs in the hippocampus impairs long-term memory formation. **(A)** To impede NR4A signaling in forebrain neurons, a tTA transgene expressed selectively in the forebrain was used to activate a dominant-negative *Nr4a* transgene (NR4ADN) under control of the tetO. **(B)** An antibody to the YFP tag on the transgenic NR4ADN protein coimmunoprecipitates endogenous NR4A2 protein from hippocampal protein extracts, confirming the ability of the dominant-negative transgenic protein to heterodimerize with NR4A protein. **(C)** In the top row, immunolabeling for the NR4ADN hemagglutinin (HA) tag (brown) with cresyl violet counterstain (purple) shows expression in the hippocampus as well as in cortex and striatum (original magnification, $\times 100$). Fluorescent immunolabeling for the YFP tag (middle row) and propidium iodide counterstaining (bottom row) illustrates transgene expression in hippocampal subregions CA1 (original magnification, $\times 250$) and the dentate gyrus (DG) (original magnification, $\times 250$) but not the amygdala (original magnification, $\times 62.5$). **(D)** NR4ADN mice have selective deficits in long-term contextual fear memory, whereas neither short-term contextual nor long-term cued fear conditioning are impaired. **(E)** No difference in 24-hour contextual fear memory performance was detected between wild-type and NR4ADN mice after 4 weeks of doxycycline (dox) treatment ($P = 0.87$, $n = 12$ mice/group). All error bars denote SEM. $*P < 0.05$. See also Supplemental Figure 2.

NR4A1 (NR4ADN) contains the DNA-binding and dimerization domains but lacks the transactivation domain (Supplemental Figure 2A), allowing it to form nonproductive dimers with all 3 NR4A proteins (25). As previously reported (25, 27), this truncated form of NR4A1 efficiently blocks NR4A-mediated transcriptional activity (Supplemental Figure 2B). We generated a transgenic mouse line expressing the NR4ADN construct under control of the tetracycline operator (tetO), which we combined with the CaMKII-tetracycline transactivator (CaMKII-tTA) transgene to achieve expression selectively within postnatal excitatory forebrain neurons (ref. 30 and Figure 2A). Endogenous NR4A2 protein was immunoprecipitated from hippocampal extracts with antibodies for the yellow fluorescent protein (YFP) tag of the NR4ADN protein

(Figure 2B), showing that the dominant-negative protein interacts with NR4A proteins in vivo. Transgene expression was restricted in the forebrain to the striatum, sparse cortical areas, and subregions of the hippocampal formation (CA1 and the dentate gyrus) (Figure 2C). Transgene expression was not observed in the amygdala or in area CA3 of the hippocampus (Figure 2C).

NR4A dominant-negative transgenic mice display impaired long-term hippocampus-dependent memory. To investigate the role of NR4A signaling in memory formation, we examined associative memory in NR4ADN mice using the fear-conditioning paradigm (Figure 2D). As discussed previously, the contextual version of this task requires hippocampal function. In contrast, long-term associative memory performance for a cue-induced fear response requires the

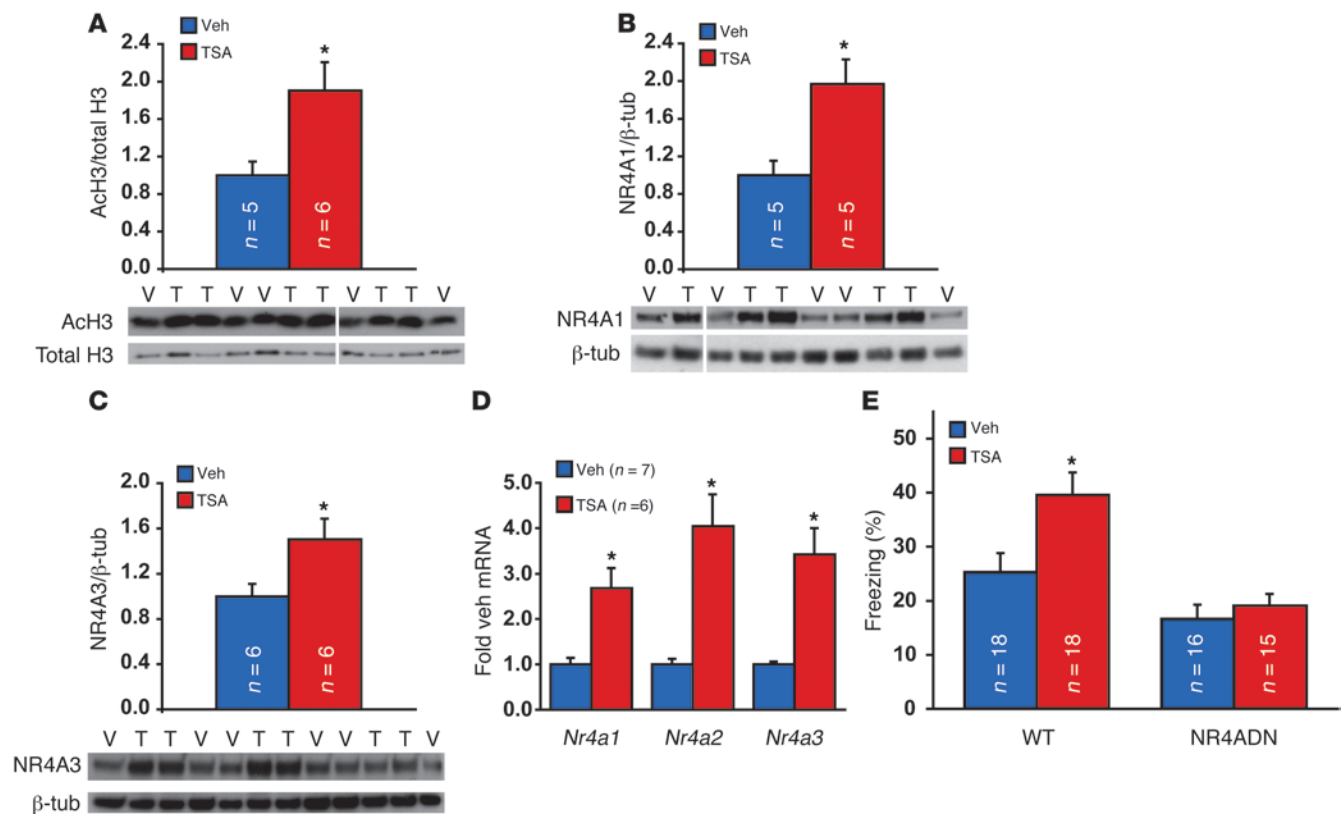


Figure 3

The ability of an HDAC inhibitor to enhance memory is blocked by the *Nr4a* dominant-negative transgene. (A) Intrahippocampal injection of the HDAC inhibitor TSA (T) enhances acetylation of histone H3 (AcH3), as illustrated at 1 hour after TSA injection after training. Veh/V, vehicle. (B and C) Intrahippocampal TSA also increases expression of the (B) NR4A1 and (C) NR4A3 protein at the same time point. (D) Intrahippocampal TSA increases mRNA levels for each of the 3 *Nr4a* genes at 1 hour after injection. (E) Injection of TSA into hippocampi after training enhances 24-hour contextual fear memory of wild-type mice but fails to enhance memory of NR4ADN littermates. Error bars represent SEM. * $P < 0.05$. See also Supplemental Figure 3. Lanes were run on the same gel but were noncontiguous (white lines).

same behavioral output, freezing, without requiring hippocampal function (14). NR4ADN mice had reduced freezing in a 24-hour test of long-term contextual memory ($P = 0.03$). In contrast, cued fear memory was unaffected in NR4ADN mice ($P = 0.81$), suggesting that the deficit in long-term contextual memory performance is likely due to defects in hippocampal function. Long-term memory deficits could result from either impaired learning or impaired memory consolidation, but performance in short-term memory tests requires learning without requiring transcription-dependent memory consolidation processes (1). Therefore, we examined short-term contextual fear memory in NR4ADN mice to test whether the *Nr4a* family of transcription factors contributes to learning or memory consolidation (Figure 2D). NR4ADN mice displayed levels of freezing in a 1-hour memory test that were similar to those of wild-type littermates ($P = 0.71$), demonstrating that NR4ADN mice are capable of learning the fear-conditioning task but have a reduced ability to retain the memory. Thus, it appears that *Nr4a* family function is involved in the consolidation of long-term contextual memory.

Memory deficit in NR4A mutant mice requires adult transgene expression. Regulation of the NR4ADN transgene by CaMKII- ϵ TA led to transgene expression selectively in postnatal neurons (Figure 2), but it is possible that the defect in long-term memory observed in these mice is due to a developmental requirement for *Nr4a* family

function or is a consequence of transgene insertion. To address these possibilities, we reared NR4ADN mice and wild-type mice in the absence of doxycycline, as before, and then placed the mice on a doxycycline diet from weaning until 2 months of age. A 1-month-long treatment with doxycycline was sufficient to suppress transgene expression (Supplemental Figure 2E). After transgene suppression, 24-hour contextual fear memory performance in NR4ADN mice was equivalent to that in wild-type littermates that were also fed an identical doxycycline diet ($P = 0.68$, Figure 2E). These data are consistent with a requirement for *Nr4a* family function in the adult mouse brain, rather than a memory defect as a result of a developmental role of *Nr4a* family members or an effect caused by transgene insertion.

Intrahippocampal HDAC inhibitor treatment increases Nr4a gene expression. Memory enhancement by pharmacologically increasing histone acetylation with HDAC inhibitors requires CREB-mediated gene expression, and HDAC inhibitor treatment was observed to increase expression of only 2 out of 13 CREB target genes, *Nr4a1* and *Nr4a2* (13). This increase in *Nr4a* gene expression was accompanied by increased histone acetylation at the promoters of these genes (13). Additionally, intrahippocampal injection of siRNA targeting *Nr4a2* attenuates the enhancement in memory observed with deletion of HDAC3 (22). These data suggest that *Nr4a* family gene expression may be an important



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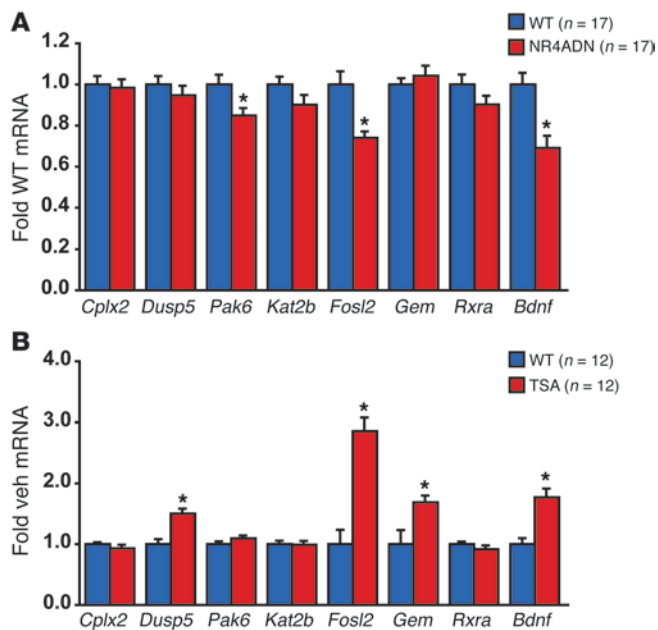


Figure 4 Common gene targets are impaired by NR4ADN expression and increased by TSA treatment. **(A)** NR4ADN transgenic mice have reduced expression of several putative *Nr4a* target genes, including *Bdnf*, *Fosl2*, and *Pak6*. **(B)** Intrahippocampal TSA treatment increases expression of several genes, including *Fosl2* and *Bdnf*. Error bars represent SEM. * $P < 0.05$. See also Supplemental Figure 4.

component of the enhancement in memory by HDAC inhibitors. We tested this hypothesis by examining whether the HDAC inhibitor trichostatin A (TSA) is capable of increasing memory in NR4ADN mice. Injection of the HDAC inhibitor TSA into the dorsal hippocampus caused increased acetylation of histone H3 (Figure 3A). This increase in histone acetylation was accompanied by a similar rise in the protein level for NR4A1 (Figure 3B) using a specific antibody against NR4A1. Furthermore, these changes at the protein level were accompanied by increased transcript levels for all 3 *Nr4a* genes (Figure 3D). Thus, our results agree with previous findings that HDAC inhibitor treatment enhances *Nr4a* gene expression (13).

Memory enhancement by HDAC inhibitors is blocked in NR4A mutant mice. The observation that *Nr4a* family members play a role in memory consolidation suggests that increased *Nr4a* gene expression after HDAC inhibitor treatment (Figure 3, B and C, and ref. 13) may contribute to memory enhancement produced by these drugs. Because NR4ADN mice retained some degree of long-term memory (Figure 2D), we were able to ask whether HDAC inhibitors are capable of enhancing memory in these mutant mice (Figure 3D). We injected the HDAC inhibitor TSA directly into the hippocampus immediately after contextual fear conditioning. Consistent with previous findings (13), intrahippocampal TSA injection after training increased long-term contextual fear memory in wild-type littermates of NR4ADN mice (Figure 3E, $P = 0.038$). However, TSA did not enhance memory in NR4ADN mutant mice ($P = 0.99$). Thus, the function of *Nr4a* family transcription factors is required for memory enhancement caused by HDAC inhibitors, suggesting that NR4A NRs contribute to this enhancement.

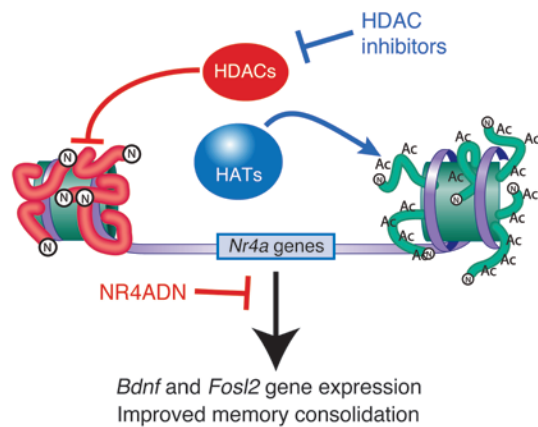
NR4ADN transgenic mice have reduced expression of NR4A target genes. To identify targets of NR4A signaling that could contribute to the memory deficit observed in NR4ADN mutant mice, we surveyed gene expression using a high-throughput qPCR approach. A 224-member gene panel was selected based on previous microarray studies after learning and neuronal activity. Using this approach, we identified 8 potential NR4A targets (Supplemental Figure 3) that we then pursued by low-throughput qPCR. We found 3 genes that were clearly impacted by NR4ADN expression (Figure 4A). Each of these genes (*Bdnf*, *Fosl2*, and *Pak6*) has potential NR4A-binding sites in the proximal promoter region, and *Bdnf* has previously been identified as a direct target of NR4A2 (31). Thus, these genes may be part of the functionally relevant output of NR4A signaling during memory formation.

Because our pharmacological studies in NR4ADN mice suggest that it is likely that TSA enhances memory by potentiating expression of NR4A target genes, we chose to further refine the list of potentially relevant targets of NR4A signaling in memory storage by examining potential NR4A target genes after TSA treatment (Figure 4B). TSA treatment increased expression of 4 out of the 8 examined genes. Both *Fosl2* and *Bdnf* were common targets of NR4A signaling and TSA treatment, suggesting that these genes might be downstream effectors for NR4A signaling that contribute to the function of NR4A signaling in memory formation (Figure 4C).

Discussion

In this work, we found that the NR gene expression increases in the hippocampus in the hours after learning a hippocampus-dependent long-term memory task. Although these NRs span multiple subfamilies, all 3 of the NR4A orphan NRs show increased expression after fear conditioning. Our results are consistent with previous reports suggesting *Nr4a1* and *Nr4a2* expression is induced by spatial and contextual exploration (19, 20). Furthermore, we found that blocking NR4A signaling in forebrain neurons, including in the hippocampus, selectively impairs long-term hippocampus-dependent contextual fear memory, without impacting short-term contextual fear memory or hippocampus-independent cued fear memory. These findings support previous suggestions that *Nr4a* function may contribute to memory formation. Further, we have confirmed that memory enhancement by HDAC inhibitors is accompanied by increased expression of *Nr4a* genes and found that blocking NR4A signaling prevents the HDAC inhibitor TSA from improving long-term memory. Finally, we identified *Fosl2* and *Bdnf* as candidate genes at the critical junction between NR4A signaling and HDAC inhibitors, as these genes have reduced expression when NR4A signaling is blocked and increased expression when an HDAC inhibitor is infused into the hippocampus (Figure 5).

We found that 13 different NR genes have increased expression after contextual fear conditioning. These NRs can be broadly divided into the group: NR4A, TLX, ERR, LXR, PPAR, RAR, and RXR. As discussed earlier, previous studies provided the suggestion that the NR4A group may contribute to memory storage (5–11), and we have provided compelling evidence supporting this possibility in this article. Yet, there is also evidence that the other NR groups identified in this study may also contribute to memory storage. For instance, TLX regulates the proliferation of adult neural stem cells, and conditional knockout of *Tlx* in the adult brain causes a defect in neurogenesis and spatial memory (32).

**Figure 5**

NR4A signaling contributes to memory formation and enhancement by HDAC inhibitors. HDAC inhibitors increase *Nr4a* gene expression, and blocking NR4A signaling prevents memory enhancement by HDAC inhibitors, suggesting a model in which NR4A target genes contribute to memory enhancement by HDAC inhibition. In this figure, arrows represent processes that stimulate gene expression and enhance memory formation. In contrast, blunt ends signify pathways that repress gene expression and limit memory formation. Nucleosomes are indicated by the green barrels that are encircled by the gray ribbon, which illustrates promoter DNA. Acetylation (ac) of the histone proteins that constitute the nucleosome (N) is dictated by a dynamic equilibrium between HDAC and HAT activity. Impairing HAT activity would be predicted to reduce *Nr4a* gene expression and impair memory formation. As illustrated in this study, blocking HDAC activity increases *Nr4a* gene expression and enhances memory formation. Also, inhibiting the function of NR4A proteins using a dominant-negative protein blocks memory enhancement by HDAC inhibition and impedes expression of several putative NR4A target genes. The increase in *Nr4a* gene expression observed after TSA injection after training is accompanied by increased expression of the putative NR4A target genes, *Bdnf* and *Fosl2*, two memory-associated genes that may contribute to the molecular mechanism of memory enhancement by HDAC inhibitors.

Perhaps surprisingly, contextual fear memory was not impacted by *Tlx* deletion. *ERRα* and *ERRβ* both show increased expression after fear conditioning. Because ERRs bind to many of the same targets as the ER and estrogen signaling contributes to memory formation (6, 9), the relationship between this increase in ERR expression and estrogen signaling may be an interesting area of future study. Alternatively, the role played by ERRs in mitochondrial function and energy metabolism (33) may suggest that these processes contribute to learning and memory as has been suggested elsewhere (34). LXR (35) and PPAR agonists (36) have been reported to improve learning and memory deficits in Alzheimer disease mouse models, suggesting that defects in signaling for these 2 classes of NRs may contribute to pathological memory deficits. RXR and RAR both respond to retinoid acid signaling, which has been previously linked to learning and memory. Depletion of vitamin A, the dietary source of retinoids, leads to defects in synaptic plasticity (37) and hippocampus-dependent memory (8) that are acutely reversible by supplementing retinoids. Additionally, knockout mice for *RARβ* and *RXRγ* show abnormalities in hippocampus-dependent memory and synaptic plasticity (10). Therefore, it is possible that the changes in RXR and RAR expression observed in this study could contribute to memory by altering retinoid sensitivity after learning.

Increased expression of *Nr4a* genes after learning is consistent with these genes being important activity-dependent targets of CREB (24), a transcription factor involved in long-term memory formation (38). Additionally, memory enhancement by HDAC inhibitors requires the interaction between CREB and the histone acetyltransferase (HAT) CREB-binding protein (CBP) as well as being accompanied by increased gene expression for *Nr4a1* and *Nr4a2* (13). In other systems, NR4A signaling is mediated by redundant roles of these 3 transcription factors (see ref. 39 for review). For these reasons, we undertook an analysis of NR4A function in memory formation using a well-tested dominant-negative strategy that was developed for the study of NR4A function in other physiological contexts (25, 27). Using this approach, we found that impeding NR4A function impairs long-term memory formation. We have now found that impeding NR4A signaling blocks the ability to enhance memory by HDAC inhibitors, suggesting that this family of NRs may be a useful target for modulating memory function.

Previous studies have found that *Nr4a1* gene expression increases in hippocampal area CA1 after contextual fear conditioning (19). Additionally, *Nr4a2* gene expression increases within both CA1 and CA3 after spatial exploration (20). In this study, transgenic NR4A dominant-negative protein was expressed under control of the CaMKII promoter, limiting expression to forebrain excitatory neurons, including those in the hippocampus. Behavioral deficit in this transgenic mouse line supports a role for NR4A signaling in hippocampal memory consolidation. Transgene expression in this mouse line was fortuitously restricted to neurons within hippocampal area CA1 and the dentate gyrus. The HDAC inhibitor injection protocol used in this study targets hippocampal area CA1 (13, 40, 41). Together, our data suggest that NR4A proteins act within excitatory pyramidal neurons in area CA1 to support memory consolidation and the enhancement in memory achieved with HDAC inhibitor treatment.

An important future direction is the identification of the mechanism by which NR4A signaling supports memory formation, which is likely to be through the activation of downstream target genes. We found that mice expressing the NR4ADN transgene have impaired expression of genes *Pak6*, *Fosl2*, and *Bdnf*. Both *Fosl2* and *Bdnf* are also potentiated by HDAC inhibitor treatment. *Fosl2*, also known as *Fra2*, is a member of the AP-1 family of transcription factors, a family that is known to be important for memory storage (42). FOSL2 is a long-lasting FOS-related antigen that, like Δ FosB, can be retained long after induction (43), suggesting that the persistence of this protein during memory storage may be an interesting subject of future inquiry. *Bdnf* is a known *Nr4a* target gene (31) that contributes to memory formation (44). The specific *Bdnf* promoter 1 that is impaired in NR4ADN mice is activated in a second, late response to neuronal stimulation (45). Thus, regulation of this promoter by NR4A signaling may represent an important mechanism governing *Bdnf* expression in later waves of transcription after learning, an intriguing idea in light of the growing appreciation that *Bdnf* contributes to memory at late time points after learning (44). The impact of the NR4ADN transgene on *Bdnf* expression provides a direct link between NR4A signaling and an effector gene known to be involved in memory formation (44). Additionally, *Bdnf* expression is potentiated by TSA treatment, suggesting that this gene might contribute to the enhancement in memory observed with TSA administration.



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The present findings are especially exciting because *Nr4a* polymorphisms have been identified in patients with schizophrenia (46), and *Nr4a* gene expression is reduced in patients with schizophrenia (47). Thus, impaired *Nr4a* function may contribute to the cognitive impairments that accompany this psychiatric disorder. Agonists for other NRs, such as PPAR and LXR, have shown promise for the treatment of Alzheimer disease (48). Over the last several years, small molecules that increase the activity of one or more NR4A proteins have been identified (49, 50). Our data suggest that the therapeutic value of these NR4A agonists is worthy of further investigation. Future approaches to ameliorate the cognitive impairment associated with neuropsychiatric disorders will greatly benefit from the knowledge that *Nr4a* family function is required for memory enhancement by HDAC inhibitors. The specific requirement for *Nr4a* family function in memory enhancement by HDAC inhibitors promises to provide more refined targets for memory improvement than would be possible with even the most specific HDAC inhibitors.

Methods

Subjects. Mice were maintained under standard conditions, with food and water available ad libitum. Adult mice (2 to 6 months of age) were kept on a 12-hour-light/12-hour-dark cycle, with lights on at 7 AM. All behavioral and biochemical experiments were performed during the light cycle, with training and testing at approximately 10 AM.

The HA-tagged truncated *Nr4a1* plasmid (27) (obtained from Jacques J. Tremblay, CRCHUQ, Quebec City, Quebec, Canada) was subcloned in-frame with the YFP tag in the pcDNA6.2 N-YFP-GW TOPO vector (Invitrogen, no. 45-1903). The YFP-HA-NR4ADN fragment was ligated into the EcoRV site of MM400 to place the YFP-NR4ADN into a hybrid intron structure under control of the tetO (30). MM400-YFP-HA-NR4ADN was purified by CsCl gradient centrifugation. The 2.7-kb tetO-YFP-HA-NR4ADN transgene fragment was injected into pronuclei of C57BL/6 zygotes (Transgenic and Chimeric Mouse Facility at the University of Pennsylvania). Founders were crossed to C57BL/6J mice bearing the CaMKII-*tTA* (line B) transgene (30). Genotyping was performed by Southern blotting using transgene-specific probes and/or PCR.

Behavior. Fear conditioning was performed as previously described (13), with handling for 3 days prior to conditioning. Briefly, the conditioning protocol entailed a single 2-second, 1.5-mA foot shock, terminating at 2.5 minutes after placement of the mouse in the chamber. Testing was performed at 1 hour or 24 hours after training over a 5-minute interval. For experiments involving TSA injection, 0.75-mA shock intensity was used to avoid a ceiling effect. Cued fear conditioning was performed as described for contextual fear conditioning, except that a 30-second cue (white noise) co-terminated with a 0.75-mA foot shock. For cued testing, mice were placed into a new context (a distinct conditioning chamber with smooth flat flooring, altered chamber dimensions, and a different odorant) for 2-minutes in the absence of the conditioned stimulus (white noise), followed by a 3-minute presentation of the auditory conditioned stimulus. Conditioning was quantified by measuring freezing behavior, the absence of nonrespiratory movement (14), using automated scoring software (Clever Systems).

Intrahippocampal TSA injection. Based on previous work (13, 40, 41), bilateral 22-gauge guide cannula were implanted 1 week prior to training at the following coordinates: anteroposterior, -1.7 mm; mediolateral, \pm 1.5 mm; 1.5 mm dorsoventral. Injection cannula extended 0.7 mm below the guide cannula. TSA (16.5 mM, AG Scientific) or vehicle (50% ethanol) was injected at a rate of 0.5 μ l/min for 1 minute immediately after training.

RNA preparation. Hippocampal dissections were performed on ice after conditioning alternating between control and experimental groups. RNA was prepared using a modified TRIzol RNA extraction, followed by RNeasy (Qiagen) purification and DNA-free (Ambion) DNase treatment. RNA concentration was ascertained using a NanoDrop spectrophotometer (ThermoFisher Scientific).

cDNA synthesis. Real-time PCR template cDNA was produced using the RETROscript Kit (Ambion). For each reaction, 1 μ g total RNA was added to a 20 μ l total reaction volume composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 5 mM dithiothreitol, 500 μ M each dNTP, 5 μ M random decamer, 10 units RNase inhibitor, and 100 units MMLV-RT. Control reactions were performed lacking template or reverse transcriptase. Reactions were performed at 44°C for 1 hour, followed by heat inactivation at 100°C for 10 minutes. Reactions were diluted to 2 ng/ μ l in water to 500 μ l final volume.

Real-time RT-PCR. High-throughput qPCR was performed as described previously (17). Briefly, 10 ng cDNA was assayed using multiplexed TaqMan qPCR probes. Data were normalized to 18S rRNA, and *t* tests were performed against the appropriate circadian control group. Subsequent, low-throughput qPCR was performed on the ABI7500 Fast or Viia7 Real-Time PCR system using 3 separate housekeeper genes for normalization (*Gapdh*, *Tuba4a*, and *Hprt*). Relative quantification of gene expression was based on the ABI users' bulletin using a $\Delta\Delta$ Ct method and described previously (13). Fold difference in mean value for biological replicates is presented, and all samples are distinct biological replicates.

Immunolabeling. For immunolabeling experiments, mice were transcardially perfused with 4% PFA, and 30- μ m coronal cryostat sections were prepared. Sections were permeabilized in 0.1% Triton X-100 and then incubated in 1% H₂O₂ in PBS for 15 minutes at room temperature. Sections were given 3 washes for 5 minutes each before being blocked in PBS with 5% preimmune serum and 0.3% Triton X-100 for 50 minutes at room temperature. Sections were triple washed for 5 minutes each in PBS and then incubated overnight at 4°C in PBS with 2% preimmune serum (same species as that of secondary antibody; Jackson ImmunoResearch Inc.), 0.3% Triton X-100, and primary antibody (anti-GFP, 1:1,000, Invitrogen no. A11122; anti-HA, 1:1,000, Roche clone 3F10). Sections were triple washed for 5 minutes each in PBS, followed by incubation in biotinylated secondary antibody for 2 hours at room temperature in PBS. For HA immunohistochemistry, biotinylated goat anti-rat antibody (1:1,000, Jackson ImmunoResearch Inc.) was used as secondary antibody, and biotinylated donkey anti-rabbit antibody (1:1,000, Jackson ImmunoResearch Inc.) was used for the remaining labeling. Three 5-minute washes in PBS were followed with incubation for 1.5 hours at room temperature in Vectastain ABC solution (Vector Laboratories). Sections were again triple washed in PBS, incubated for 10 to 15 minutes in 0.2 mg/ml 3,3'-diaminobenzidine in PBS, and then triple washed in PBS with sodium azide (0.01%). Stained sections were mounted onto glass microscope slides in 0.7% gelatin. Sections were examined and digitally photographed through a light microscope. For immunofluorescence, Alexa Fluor 488-coupled chicken anti-rabbit IgG (1:5,000; Invitrogen) was used as secondary antibody. After incubation in fluorescent antibody, sections were washed 3 times in PBS for 5 minutes each and then mounted on to glass slides in 50% glycerol/PBS containing 20 μ g/ml propidium iodide. Images were captured using a Leica (Wetzlar) TCS SP2 confocal microscope.

Coimmunoprecipitation. Mice were cervically dislocated 30 minutes after placement in a novel context. Hippocampi were dissected and flash frozen on dry ice. Hippocampi were homogenized in 1 ml of hypotonic lysis buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, plus protease and phosphatase inhibitors [Sigma-Aldrich]), followed by rocking at 4°C for 30 minutes, and then centrifugation at 1,000 \times g



for 15 minutes at 4°C to pellet nuclei. Nuclear pellets were resuspended in 300 μ l E1A lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 5 mM EDTA, plus protease and phosphatase inhibitors [Sigma-Aldrich]) and gently rocked at 4°C for 30 minutes. Nuclear lysates were centrifuged at 1,000 \times g for 15 minutes and 4°C to pellet nuclear debris. Supernatants were diluted to 1 ml in E1A lysis buffer containing 1 μ g biotinylated anti-GFP (Invitrogen) and gently rocked overnight at 4°C. For immunoprecipitation, 50 μ l of Pierce streptavidin magnetic beads (Thermo) were then added to the protein solution on the following day. Beads were washed 3 times in E1A buffer, and antigens were eluted in 30 μ l of 0.1 M glycine (pH 2.5).

Western blotting. NuPage SDS loading buffer and 2-mercaptoethanol (Invitrogen) were added to samples prior to a 10-minute incubation at 100°C. Proteins were resolved by polyacrylamide electrophoresis on a NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen) for Western blotting. Blocking was performed with 5% milk. Washes and primary antibody incubations were performed in either TBS-0.1% Tween-20 (anti-Ach3k9/14, 1:5,000, Millipore 06-599; anti-H3, 1:5,000, Abcam ab1791; anti- β -tubulin, 1:20,000, Sigma-Aldrich T4026; anti-NR4A1, 1:1,000, eBiosciences 14-5965; anti-Nr4a3, 1:1,000, Abcam ab41918) or PBS-0.1% Tween-20 (anti-NR4A2, 1:2,000, Santa Cruz Biotechnology Inc. SC991). Three 5-minute washes were followed by incubation in PBST with HRP-conjugated goat anti-rabbit secondary antibody (1:1000, Santa Cruz Biotechnology Inc.). The blot was washed 3 times in PBST for 5 minutes each and treated with ECL Western Blotting Detection Reagent (GE Healthcare) for chemiluminescence detection. Membranes were then exposed to film for 1 to 10 minutes and developed for analysis.

Statistics. Data are expressed as mean \pm SEM. For behavioral experiments, ANOVAs were performed, followed by Student-Newman-Keuls post-hoc tests. Gene expression survey of NRs was analyzed with 2-tailed *t* tests. Fol-

low-up real-time qPCR data were analyzed with ANOVAs to detect overall effects of training and nonparametric Kruskal-Wallis ANOVAs for planned comparisons to home cage controls. High-throughput qPCR data to detect NR4ADN target genes was performed using ANOVAs with training and genotype as factors. For all statistical tests, *P* < 0.05 was considered significant. Experimenters were blind to genotype, and genotypes were confirmed after experiments were completed.

Study approval. All animal care and experiments were consistent with NIH guidelines and approved by the Institutional Care and Use Committee at the University of Pennsylvania.

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