Brief Communication

Differential roles for *Nr4a1* and *Nr4a2* in object location vs. object recognition long-term memory

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Nr4a1 and *Nr4a2* are transcription factors and immediate early genes belonging to the nuclear receptor Nr4a family. In this study, we examine their role in long-term memory formation for object location and object recognition. Using siRNA to block expression of either *Nr4a1* or *Nr4a2*, we found that *Nr4a2* is necessary for both long-term memory for object location and object recognition. In contrast, *Nr4a1* appears to be necessary only for object location. Indeed, their roles in these different types of long-term memory may be dependent on their expression in the brain, as NR4A2 was found to be expressed in hippocampal neurons (associated with object location memory) as well as in the insular and perirhinal cortex (associated with object recognition memory), whereas NR4A1 showed minimal neuronal expression in these cortical areas. These results begin to elucidate how NR4A1 and NR4A2 differentially contribute to object location versus object recognition memory.

[Supplemental material is available for this article.]

It is well established that long-term memory (LTM) formation requires transcription (for review, see Alberini 2009). Transcription regulated specifically by cAMP responsive element binding protein (CREB) has been shown to be essential for long-term memory (Bourtchuladze et al. 1994; Yin et al. 1994; Guzowski and McGaugh 1997; Pittenger et al. 2002; Sekeres et al. 2010; but see Balschun et al. 2003). Two CREB-dependent immediate early genes that have been implicated in LTM are *Nr4a1* and *Nr4a2* (Pena de Ortiz et al. 2000; von Herten and Giese 2005a,b; Colon-Cesario et al. 2006). *Nr4a1* (*Nur77*) and *Nr4a2* (*Nurr1*) are members of the nuclear steroid/thyroid hormone receptor superfamily that bind in an apparently ligand-independent manner to Nerve Growth Factor1-B (NGFI-B) response elements (Baker et al. 2003; Wang et al. 2003).

Expression of both *Nr4a1* and *Nr4a2* has been shown to increase in the hippocampus following learning. *Nr4a1* expression increased in the CA1 region of the hippocampus during context shock memory consolidation, and *Nr4a2* increased in CA1 and CA3 pyramidal cell layers of the rat hippocampus following a spatial discrimination task (Pena de Ortiz et al. 2000; von Herten and Giese 2005a,b; Keeley et al. 2006; Hawk and Abel 2011). An exception to these findings was a study from Malkani and Rosen (2000) who found increased *Nr4a1* expression in the cortex and amygdala following context shock memory consolidation, but failed to see a change in area CA1 of the hippocampus. These patterns of expression suggest a role for the Nr4a family in learning and memory.

Transcription of both *Nr4a1* and *Nr4a2* appear to be regulated by chromatin modification via histone acetylation and deacetyla-

tion. Histone deacetylase (HDAC) activity was shown to interfere with formation of the pre-initiation complex at the *Nr4a1* promoter, suggesting that acetylation is necessary for its transcription (Fass et al. 2003). In addition, during memory consolidation, the HDAC inhibitor Trichostatin A (TSA) maintained the expression of both *Nr4a1* and *Nr4a2* during memory consolidation, without maintaining the expression of several other CREB-dependent genes (Vecsey et al. 2007).

We recently showed that HDAC3 may modulate long-term memory formation in an *Nr4a2*-dependent manner (McQuown et al. 2011). Deletion of HDAC3 in the dorsal hippocampus transformed a subthreshold training event that normally does not result in LTM into an event that led to robust LTM. Deletion of HDAC3 also facilitated *Nr4a2* expression following subthreshold training. In contrast, the HDAC3 deletion had no effect on *Nr4a2* expression in naive-handled mice, suggesting that the increase in *Nr4a2* required an activity-dependent event. Notably, siRNA knockdown of *Nr4a2* in animals with HDAC3 deletions prevented the enhanced LTM that occurred following the subthreshold training (McQuown et al. 2011). Together, these studies suggest that *Nr4a1* and *Nr4a2* are key target genes regulated by acetylation during long-term memory formation.

The studies discussed above have demonstrated an important role for *Nr4a1* and *Nr4a2* in long-term memory formation. They also indicate that expression of these genes during consolidation is regulated by chromatin modification through histone acetylation and deacetylation. The current study aimed to further understand the role of these genes in long-term memory formation.

To investigate the role of *Nr4a1* and *Nr4a2* in learning and memory processes, we examined the basal and activity-dependent levels of NR4A1 and NR4A2 proteins in brain regions involved in learning and memory. We chose to examine regions involved in object location memory and object recognition memory, as the

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.026385.112>.

training for these tasks is the same, but the testing depends on different brain regions. (Bermudez-Rattoni et al. 2005; Balderas et al. 2008; Roozendaal et al. 2010; Haettig et al. 2011). We performed immunohistochemistry on 20- μ m coronal sections of formaldehyde fixed brain tissue from adult male mice to examine expression of *NR4A1* and *NR4A2* in the hippocampus as well as the insular, perirhinal, and entorhinal cortices. We also used transient siRNA knockdown to specifically examine the roles of *Nr4a1* and *Nr4a2* in learning and memory as measured by object location and novel object recognition memory tasks. The findings presented here provide new insight into how *Nr4a1* and *Nr4a2* regulate memory formation in different tasks utilizing different brain regions.

We found that NR4A1 protein was strongly expressed throughout the hippocampus, including the CA1–CA3 regions (Fig. 1B–D). Notably, in addition to hippocampal expression (Fig. 1H–J), NR4A2 showed strong expression in discrete foci within cortical layer VI (Allen Brain Atlas coordinates: -2.25 to -5.25 ventral to bregma) (Fig. 1K,L). Double labeling for NR4A2 and NeuN showed that cortical NR4A2 is expressed in neurons (Fig. 1L). These findings demonstrate that both NR4A1 and NR4A2 were expressed in the hippocampus, whereas NR4A2 also exhibited strong expression in cortical layer VI.

Next we examined changes in expression of NR4A1 and NR4A2 following training for an object memory task that activates these brain regions (Bermudez-Rattoni et al. 2005; Balderas et al. 2008; Roozendaal et al. 2010; Haettig et al. 2011). Adult male C57Bl/6J mice were habituated to the context for 4 d and then on the fifth day were returned to the same context and either exposed to two identical objects for 10 min (trained) or without objects (habituated). Preliminary time-course studies showed that

Nr4a2 mRNA levels increased for 60 min following a learning event, and Jo et al. (2009) demonstrated that NR4A2 protein is stable for >2 h. Therefore, 2 h after training animals were perfused and formaldehyde-fixed brains were collected for immunohistochemistry. The NR4A1 and NR4A2 antibodies we used for immunohistochemistry are directed against the variable N-terminal region of the two proteins, and the specificity of the antibodies was verified by Western blot analysis (Supplemental Fig. 1).

We quantified NR4A1 and NR4A2 expression in area CA1 of the hippocampus and cortical layer VI of the insular, perirhinal, and entorhinal cortices. Following training, there were significant 40% increases in both NR4A1- (habituated: 1.03 ± 0.07 $N = 23$ vs. trained: 1.43 ± 0.07 $N = 31$, $P = 0.0003$) (Fig. 2B) and NR4A2-immune-positive cells in area CA1 (habituated: 1.00 ± 0.19 $N = 12$ vs. trained: 1.39 ± 0.11 $N = 16$, $P = 0.04$) (Fig. 2C). There was no significant change in cortical NR4A1-immune-positive cells (habituated: 1.00 ± 0.36 $N = 23$ vs. trained: 0.72 ± 0.15 $N = 26$, $P = 0.24$) (Fig. 2D). In addition, we found a significant twofold increase in cortical NR4A2-immune-positive cells in mice that received a 10-min training compared with habituated animals (habituated: 1.00 ± 0.12 $N = 21$ vs. trained: 1.96 ± 0.19 $N = 27$, $P = 0.0002$) (Fig. 2E). Representative images of hippocampal NR4A1 (Fig. 2F,G) and hippocampal (Fig. 2H,I) and cortical NR4A2 (Fig. 2J,K) are shown.

To further investigate the role of *Nr4a1* and *Nr4a2* in learning and memory, we used small interfering (si) RNAs to transiently knock down expression of *Nr4a1* and *Nr4a2*. Targeted SMART pool siRNAs (Dharmacon) were prepared with jetSI (Polyplus Transfection) at a final concentration of 4 μ M before injection. A nontargeting control siRNA with an RNA-induced silencing complex (RISC)-free modification was used as a control (siRISC). Mice

received intrahippocampal infusions of RISC-free control, *Nr4a1*, or *Nr4a2* siRNA directly into the dorsal CA1 area of the hippocampus (AP, -2.0 mm; DV, -1.5 mm; ML, ± 1.5 mm). To assay specific knockdown of each transcript, RNA was isolated from 1-mm hippocampal punches and quantitative real-time RT-PCR was performed as previously described (McQuown et al. 2011) using a Roche LightCycler:

Nr4a1 probe 93: 5'-tctgtgcc-3', forward primer: 5'-agcttggtgtgtgatgttcc-3', reverse primer: 5'-aatgcgattctgc agctctt-3';
Nr4a2 probe 2: 5'-ttctcctg-3', forward-primer: 5'-ttgcagaatgatgaacatcgaca-3', reverse primer: 5'-gttccttgagcccggtg tct-3';
 Gapdh probe: 5'-tgccgctattgg-3', forward primer: 5'-atggtgaaggtcggtgtga-3', reverse primer: 5'-aatctccactttgc cactgc-3'.

Hippocampal infusion with siRNA against *Nr4a1* significantly and specifically decreased expression of *Nr4a1* by more than twofold as compared with the RISC-free control (siRISC = 1.58 ± 0.43 , si*Nr4a1* = 0.70 ± 0.18 ; $P = 0.04$). There was no significant difference in expression of *Nr4a2* following infusion with si*Nr4a1* (1.99 ± 0.37 ; $P = 0.25$) (Fig. 3A). Infusion with si*Nr4a2* also significantly

Figure 1. NR4A1 and NR4A2 are expressed in the hippocampus and NR4A2 is also expressed in cortical layer VI extending through the insular, perirhinal, and entorhinal cortices. Representative images showing (A) Nuclear DAPI staining 4 $\times$ magnification. (B) NR4A1-immune-positive cells, 4 $\times$ magnification. (C) NR4A1-immune-positive cells in CA1 of the hippocampus, 20 $\times$ magnification. (D) NR4A1-immune-positive cells in CA2 and CA3 of the hippocampus, 20 $\times$ magnification. (E) NR4A1-immunoreactivity in cortical layer VI, showing diffuse staining. (F) Minimal colocalization of NeuN- and NR4A1-immunopositive cells. (G) Nuclear DAPI staining, 4 $\times$ magnification. (H) NR4A2-immune-positive cells, 4 $\times$ magnification. (I) NR4A2-immune-positive cells in the CA1 area of the hippocampus, 20 $\times$ magnification. (J) NR4A2-immune-positive cells in CA2 and CA3 of the hippocampus, 20 $\times$ magnification. (K) NR4A2-immune-positive cells in cortical layer VI, showing strong focal expression. (L) Colocalization of NeuN- with NR4A2-immunopositive cells. The primary antibodies were directed against NR4A1 (sc-5569, Santa Cruz), NR4A2 (sc-5568, Santa Cruz), and NeuN (MAB377, Millipore Corporation). Images were acquired with an Olympus (BX51) microscope using 4 $\times$ (A,B,G,H) or 20 $\times$ (C–F,I–L) objectives and a CCD camera (QImaging) using QCapture Pro 6.0 software (QImaging).

and specifically knocked down *Nr4a2* expression more than two-fold when compared with RISC-free control ($\text{siRISC} = 0.97 \pm 0.23$, $\text{siNr4a2} = 0.46 \pm 0.07$; $P = 0.02$). No significant difference was found in expression of *Nr4a1* following infusion with

siNr4a2 (0.72 ± 0.11 ; $P = 0.17$) (Fig. 3B). Each primer–probe set produced a single real-time RT-PCR product. In addition, there were no significant differences in *Gapdh* mRNA expression following *siRNA* knockdown (Supplemental Fig. 2).

We then used the object location memory (OLM) task to investigate the roles of *Nr4a1* and *Nr4a2* in the hippocampus. Two days prior to training, mice received hippocampal *siRNA* infusions and 48 h after infusion were trained with two identical objects for 10 min (Fig. 3C). The percentage of time spent exploring the objects during training did not differ significantly between the groups (statistics calculated using ANOVA). Twenty-four hours after training, the animals were placed in the identical context with one object moved to a novel location (OLM) (Fig. 3C). Animals that spent more time with the object in the novel location are considered to demonstrate memory for the object in the familiar location. Animals that received infusions of the control RISC-free *siRNA* exhibited memory for the familiar object and spent more time with the object in the novel location (% discrimination index [DI] = 27.7 ± 8.4 , $N = 8$) (Fig. 3D). In contrast, animals infused with *siRNA* against either *Nr4a1* or *Nr4a2* had significantly impaired memory for the familiar object (% DI for *Nr4a1* = 4.8 ± 5.5 , $N = 9$, % DI for *Nr4a2* = -6.9 ± 4.7 , $N = 10$, $P = 0.002$). These results suggest that both *Nr4a1* and *Nr4a2* are required for hippocampus-dependent long-term memory for object location.

We next examined the role of *Nr4a1* and *Nr4a2* in long-term memory in a standard novel object recognition task (ORM) (Fig. 3E). Two days prior to training, mice received intracerebroventricular (ICV) infusions (AP, -0.34 mm; DV, -2.1 mm; ML, ± 1.0 mm) of small interfering (si) RNAs to transiently knock down expression of *Nr4a1* and *Nr4a2*. In the ORM task, 48 h after *siRNA* infusion, animals previously habituated to the context were trained with two identical objects for 10 min (Fig. 3E). There were no significant differences in total exploration times between groups during training or testing (statistics calculated using ANOVA).

Twenty-four hours after training, animals were given a retention test in which one familiar object was replaced with a novel object (Fig. 3E). At testing, animals infused with *siRNA* targeted against *Nr4a2* had a significantly lower discrimination index (% DI) for the novel object (% DI = 10.1 ± 4.5 , $N = 6$; $P = 0.03$) than mice infused with either control *siRISC* (% DI = 27.88 ± 5.7 , $N = 9$) or *siRNA* directed against *Nr4a1* (% DI = 29.1 ± 2.6 , $N = 7$). There was no difference between control animals and animals infused with *siNr4a1* (Fig. 3F). The *siRNAs* targeted to *Nr4a2* resulted in a significant knockdown of NR4A2 protein in layer VI of the insular, perirhinal, and entorhinal cortices 2 h after training with objects, ($\text{siRISC} = 1.00 \pm 0.30$, $N = 13$, $\text{siNr4a2} = 0.35 \pm 0.05$, $N = 15$, $P = 0.02$) (Fig. 3H). As predicted from the lack of neuronal NR4A1 expression in cortical areas, no significant difference in cortical expression was observed following *Nr4a1* knockdown ($\text{siRISC} = 0.97 \pm 0.38$, $N = 13$, $\text{siNr4a1} = 0.83 \pm 0.33$, $N = 15$, $P = 0.38$) (Fig. 3G). These data show that cortical knockdown of *Nr4a2* impaired long-term memory for the object itself.

Both *Nr4a1* and *Nr4a2* mRNAs are highly expressed in the CA1 and CA3 regions of the hippocampus (Honkaniemi et al. 1995; Xiao et al. 1996; this study), an area critical for context and location-dependent memory formation (O'Keefe and Burgess 1999; Fanselow 2000; Maren and Holt 2000). Through a number of recent studies, it has been found that mRNA expression of *Nr4a1* and *Nr4a2* increases during memory acquisition and consolidation, suggesting that induction of these genes is integral to the formation of long-term memory (Pena de Ortiz et al. 2000; von Hertzen and Giese 2005a,b; Colon-Cesario et al. 2006). Acquisition and LTM for a spatial discrimination task in rats is disrupted

Figure 3. Knockdown of *Nr4a2*, but not *Nr4a1*, in cortical regions including the insular, perirhinal, and entorhinal cortices reduces preference for the novel object in the object recognition (ORM) task, while hippocampal knockdown of both *Nr4a1* and *Nr4a2* affects performance in the object location (OLM) task. (A) siRNA directed against *Nr4a1* significantly reduced hippocampal expression of *Nr4a1* ($P = 0.04$) without affecting *Nr4a2* ($P = 0.25$). (B) siRNA against *Nr4a2* significantly reduced hippocampal expression of *Nr4a2* ($P = 0.02$) without affecting *Nr4a1* expression ($P = 0.17$). (C) Forty-eight hours after infusion, habituated animals received 10 min of training with two identical objects. Another 24 h later they were given a retention test in which one object was moved to a novel location. (D) During a 24-h retention test, mice that received hippocampal infusions of either siNr4a1 or siNr4a2 displayed no preference in the OLM task in contrast to animals that received control RISC-free siRNA ($P = 0.002$). (E) Habituated mice received intracerebroventricular (ICV) infusions of control RISC-free, *Nr4a1*, or *Nr4a2* siRNA. Forty-eight hours later they received 10 min of training with two identical objects. Twenty-four hours later they were given a retention test where one object was replaced with a novel object. (F) During a 24-h retention test, mice that received ICV infusions of siNr4a2 displayed no preference for the novel object in contrast to animals that received control RISC-free siRNA or siNr4a1 ($P = 0.03$). (G) Intracerebroventricular infusions of *Nr4a1* siRNA did not change expression of NR4A1 in the cortex, as measured by immunohistochemistry ($P = 0.4$). (H) Intracerebroventricular infusions of *Nr4a2* siRNA significantly decreased expression of NR4A2 in the cortex, as measured by immunohistochemistry ($P = 0.02$).

by oligodeoxynucleotide knockdown of *Nr4a2* in the CA3 area of the hippocampus (Colon-Cesario et al. 2006). In this study, we showed that NR4A1 and NR4A2 protein expression is increased in the hippocampus following an object memory-training task. siRNA knock down of either of these genes affects learning and

memory in tasks relying on the hippocampus. These data demonstrate critical roles for *Nr4a1* and *Nr4a2* in the hippocampus during long-term memory formation.

Although their expression has been previously examined, little is known about the role of the *Nr4a* genes in cortex-dependent memory. Several studies have reported that *Nr4a1* mRNA is expressed throughout the cerebral cortex (Chan et al. 1993; Honkaniemi et al. 1995; Xiao et al. 1996; Zetterström et al. 1996), while *Nr4a2* mRNA is not as widespread in the CNS (Saucedo-Cardenas and Conneely 1996; Xiao et al. 1996). However, expression of *Nr4a2* mRNA in regions associated with memory acquisition and consolidation has been proposed to play a role in memory processing (Xing et al. 1997). Our findings support this, showing that following a learning event, expression of NR4A2 protein increased significantly in cortical layer VI. siRNA targeted to *Nr4a2* in the cortex led to a significant reduction in NR4A2 and impaired a cortex-dependent memory task. Together these findings suggest a critical role for NR4A2, but not NR4A1 in cortex-dependent memory formation.

While the perirhinal cortex is considered essential for poly-modal object recognition (Albasser et al. 2011), the hippocampus is believed to be necessary for object location. However, a functional interaction between the hippocampus and the perirhinal cortex is important for multisensory processing and memory (Barker and Warburton 2011). The results presented here suggest that NR4A1 is a critical signaling molecule involved in object location memory, while NR4A2 is critical for both object location and recognition memory.

Previously, we showed that *Nr4a2* was necessary for memory enhancement resulting from a focal hippocampal deletion of HDAC3 (McQuown et al. 2011). In the present study, we showed that in addition to *Nr4a2*, *Nr4a1* is critical for hippocampal memory formation. Further studies have shown that HDAC activity interferes with transcription of *Nr4a1* and an HDAC inhibitor increases expression of both *Nr4a1* and *Nr4a2* in the hippocampus (Fass et al. 2003; Vecsey et al. 2007). Therefore, further studies will be needed to answer whether HDAC3 is also a critical regulator of *Nr4a1* expression during LTM formation. We also found that cortical NR4A2 is

specifically required for long-term memory for object recognition. These data suggest that *Nr4a1* and *Nr4a2* are critical for specific types of memory and represent different mechanisms required for consolidation of long-term memory in different brain regions.

Acknowledgments

This work was supported by grants from NIMH (MH081004) and NIDA (DA025922; DA031989) to M.A.W., a predoctoral NRSA fellowship (F31MH85494) to R.M.B.; a Re-Entry into Biomedical and Behavioral Career supplement (MH081004-02S1) to S.E.M.; a predoctoral NRSA fellowship (F31DA29368) to M.M.; Biomedical Research Support (MBRS) Program, NIH Grant GM-55246 to M.F.D. and N.H. We especially thank Dr. John Guzowski for the use of his Olympus BX61 microscope and XC10 camera.

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Received March 12, 2012; accepted in revised form August 29, 2012.

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Susan E. McNulty, Ruth M. Barrett, Annie Vogel-Ciernia, et al.

Learn. Mem. 2012, **19**:

Access the most recent version at doi:[10.1101/lm.026385.112](https://doi.org/10.1101/lm.026385.112)

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