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Activation of the vitamin A–retinoic acid signaling pathway enhances memory formation

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Abstract

Retinoic acid (RA), a biologically active metabolite of vitamin A (VA), regulates gene expression through retinoic acid receptor/retinoid X receptor (RAR/RXR)-dependent transcription as their ligand and plays important roles in various biological phenomena. Dietary, pharmacological, and genetic dysfunction of the VA-RA signaling pathway impairs hippocampal synaptic plasticity, including long-term potentiation and memory, indicating that this pathway plays an essential role in synaptic plasticity and memory. However, it remains unclear whether the gain-of-function of VA-RA signaling enhances memory formation. Here, we examined the effects of gain-of-function of hippocampal VA-RA signaling on hippocampus-dependent memory and hippocampal transcription profiles. Intrahippocampal microinfusion of RAR/RXR ligands RA (ATRA) or 9-cis RA (9-cis RA), enhanced long-term contextual fear and social recognition memories without affecting short-term memory. On the other hand, microinfusion of Ro41-5253, a selective antagonist for RARα, impaired long-term memory. These observations suggest that VA-RA signaling function positively regulates memory formation. To further examine the memory-enhancing effects of VA-RA signaling, RARα transgenic mice overexpressing RARα in the forebrain were generated.. Interestingly, these mutant mice showed enhanced long-term memory even in the absence of ATRA treatment, indicating that RARα overexpression is sufficient to enhance memory. Transcriptome analysis revealed that systemic ATRA treatment and/or RARα overexpression resulted in similar hippocampal transcription profiles and changes in the expression of learning and memory-related gene sets. We also found that activation of VA-RA signaling upregulates mRNA levels of Chrm4, a muscarinic acetylcholine receptor M4. Interestingly, an intraperitoneal injection of a Chrm4 agonist enhanced memory formation. Taken together, these results suggest that hippocampal VA-RA signaling enhances memory formation. Thus, essential nutrient VA-RA functions as a memory enhancer.

Introduction

Retinoic acid (RA) is a biologically active metabolite of the essential nutrient vitamin A (VA). It plays important roles in various biological phenomena including reproduction, growth, differentiation, development, vision, and tissue homeostasis, across major organs, including the brain [1–3]. *All-trans* RA (ATRA) and *9-cis* RA bind to RA receptors (RARs) and retinoid X receptors (RXRs), respectively, functioning as ligand-dependent transcription factors. These nuclear receptors regulate the transcription of target genes by binding to RA response elements as either RAR/RXR heterodimers or RXR/RXR homodimers [1–3]. Thus, the VA-RA signaling pathway acts as a nutrient-dependent transcriptional regulatory system.

Both RARs (especially RARα) and RXRs are highly expressed in the developing and mature brain, indicating that VA-RA signaling is important for brain development, including cortical and hippocampal circuit formation and neuronal connectivity [4]. VA-RA signaling is also necessary for hippocampal plasticity and memory processes [5, 6]. Dietary VA deprivation has been shown to impair hippocampal long-term potentiation (LTP) [7], and RARβ or RARβ/RXRγ knockout mice exhibit deficits in LTP and long-term depression [8]. Consistently, we have previously shown that transgenic mice expressing a

dominant-negative RARa mutant in the forebrain impaired hippocampal LTP and hippocampus-dependent memory formation [9]. Interestingly, both dietary VA supplementation and systemic RA treatment improved memory deficits in middle-aged, aged, and Alzheimer's disease rodent models [10–13]. Taken together, these findings suggest that loss of VA-RA signaling function impairs learning and memory. However, it remains unclear whether the gain-of-function of this signaling pathway enhances memory formation in the mature brain.

Here, we investigated the effects of pharmacological gain- and loss-of-function of hippocampal VA-RA signaling on hippocampus-dependent memory. To further understand the molecular basis of these effects, we generated novel RARα transgenic mice overexpressing RARα in the forebrain, thus inducing genetic gain-of-function of VA-RA signaling. We analyzed hippocampus-dependent memory in these transgenic mice and identified candidate genes associated with positive regulation of memory formation through hippocampal transcriptional analyses of these mutant mice.

Materials and Methods

Mice

Mice were group-housed (5–6 per cage) under a 12 h light/dark schedule, and allowed ad libitum access to food and water in their home cages. All of the experiments were conducted during the light phase of the cycle in an illuminated testing room according to the Guide for the Care and Use of Laboratory Animals, Japan Neuroscience Society and Tokyo University of Agriculture (Approval number: 25).

Plasmid construction

Plasmids were constructed as previously described [9, 14, 15]. Briefly, to generate RARα cDNA, the RARα cDNA fragment corresponding to nucleotides 1–1389 was amplified by reverse transcription-polymerase chain reaction (RTPCR), using mouse brain cDNA as a template with the following primers (5' to 3'):

Forward: GGGGATCCACGCGTGGCGGCCGCATGGCCAGCAATAGCAGTTC

Reverse: GGGGAATTCACGCGTAAGCTTTCATGGGGATTGGGTGGCTG.

The resulting RT-PCR fragments were subcloned into the BamHI-EcoRI sites of pBluescript II (SK+) (Agilent Technologies, CA, USA), generating pBS-RARα. The BglII-HindIII fragment encoding RARα from pBS-RARα was then ligated with the BglII-HindIII fragment from pTRE-dnRAR [9], generating pTRE-RARα.

Generation of transgenic mice

Generation of the RARα mutant was performed as previously described [9, 14, 15]. Briefly, pTRE-RARα was digested with MluI, and the purified insert was injected into the pronuclei of C57BL/6N zygotes.

TRE-RARα founders were crossed with C57BL/6N mice (Charles River Japan, Kanagawa, Japan) and their offspring were crossed with mice (CaMKII-tTA) expressing the tTA transgene under the control of the aCaMKII promoter [16], generating the double-transgenic RARα mice. Genotyping was performed by Southern blot analysis using specific ^{32}P-labeled probes derived from the 0.5 kbp NotI-NdeI fragment containing the TRE-promoter region from pcisTRE and the 0.6 kbp EcoRI-HindIII fragment containing the tTA-coding region from pcDNA3-TetR-KRAB [15].

Biochemical experiments

Total RNA preparation and Northern blot analysis were performed as previously described [9, 14, 17, 18]. To generate a specific probe for TRE promoter-dependent transcripts, the 0.3 kbp HindIII-XbaI fragment containing the 3'-untranslated region (UTR) from pTRE-RARα was used. The filters were hybridized with a specific ^{32}P-labeled probe for the 3'-UTR, as previously described [9, 14]. Quantitative RT-PCR (qRT-PCR) was performed on an ABI PRISM 7000 using the Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) according to the manufacturer's protocol. The reaction was first incubated at 50°C for 2 min, then at 95°C for 10 min. This was followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. All measurements were performed in triplicate. GAPDH mRNA levels were used to normalize the relative expression levels of target mRNA. The following PCR primers were used (5' to 3')):

GAPDH forward: ATGGCCTTCCGTGTT CCTAC

GAPDH reverse: GCCTGCTTCACCACCTTCTT

RARb forward: TCCTGGATCAATGCCACCTC

RARb reverse: ACACGCTGGACTGTGCTCT

Akt3 forward: TGGGTTCAGAAGAGGGGAGAA

Akt3 reverse: AGGGGATAAGGTAAGTCCACATC

Chrm4 forward: ATGGCGAACTTCACACCTGTC

Chrm4 reverse: CTGTCGCAATGAACACCATCT

Cnr1 forward: AAGTCGATCTTAGACGGCCTT

Cnr1 reverse: TCCTAATTTGGATGCCATGTCTC

Adcy1 forward: GTCACCTTCGTGTCCTATGCC

Adcy1 reverse: TTCACACCAAAGAAGAGCAGG

Drug

ATRA (Sigma-Aldrich, R2625) was used as an agonist of RA receptors. Hippocampal microinfusion experiments were performed using 9-cis RA (Sigma-Aldrich, R4643) and the selective RARα antagonist Ro41-5253 (Ro41; Enzo Life Sciences, NY, USA). ATRA for intraperitoneal administration was prepared by dissolution in ethanol, after which 100 µL of the solution was mixed with 1 mL of peanut oil (Sigma-Aldrich, P2144). Ethanol was then evaporated using a vacuum centrifuge. The solution was subsequently replaced with nitrogen gas to minimize oxidation, protected from light, and stored at − 80°C until use [17]. Intraperitoneal administration was performed at a volume of 0.2 mL per 30 g of body weight. Oxotremorine sesquifumarate (OXO; Tocris Bioscience, Cat. No. 0843/100) was dissolved in saline (10 mg in 20 mL) and stored at 4°C as a stock solution. Before administration, the stock solution was diluted 100-fold with saline. OXO was administered intraperitoneally at a dose of 0.05 mg/kg body weight.

Surgery for drug microinfusion

Surgery was performed as previously described [9, 19]. Briefly, under Nembutal anesthesia and using standard stereotaxic procedures, stainless steel guide cannulae (22 gauge) were implanted into the dorsal hippocampus at the following coordinates: anteroposterior, − 2.0 mm; mediolateral, ± 1.4 mm; dorsoventral, − 1.6 mm from bregma. For local infusion, 10 mM stock solutions were prepared by dissolving ATRA, 9-cis RA, and Ro41 in dimethyl sulfoxide (DMSO). The stock solutions were then diluted with phosphate-buffered saline (PBS) immediately before use. ATRA was used at 1 µM, 9-cis RA was used at 10 µM, and Ro41 was used at 1 µM for the social recognition task and 10 µM for contextual fear conditioning. Drugs were bilaterally infused into the dorsal hippocampus at a volume of 0.5 µL per side, at a rate of 0.25 µL/min. The injection cannula was left in place for 2 min after infusion.

Infusion was histologically confirmed at each site using hematoxylin-eosin staining. Only mice in which bilateral hippocampal infusion was confirmed histologically were included in the data analysis.

RNA-sequencing (RNA-Seq)

RNA-seq was performed as previously described [14, 20]. Total hippocampal RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Each experimental group underwent RNA-seq analysis as biological triplicates (n = 3). After RNA quality check (all RIN values > 8.2) with the RNA Nano Kit (Agilent Technologies) on the Agilent Bioanalyzer (Agilent Technologies), 1 µg of total RNA was used to prepare cDNA libraries with the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA), following the manufacturer's instructions. The derived cDNA libraries were analyzed on an Agilent Bioanalyzer with DNA 1000 Kit and quantified by qPCR, using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA). Twelve libraries with unique barcodes were pooled in three lanes and clusters were generated on a cBot (Illumina) to obtain 100 bp single reads in a HiSeq 2500 sequencer (Illumina). Generation of demultiplexed FASTQ files was performed using bcl2fastq ver. 2.18 (Illumina). Filtering, mapping, and differential expression analysis

were performed using the CLC Genomics Workbench software ver. 9.5 (Qiagen). The raw sequence reads were filtered to exclude adapter sequences, ambiguous nucleotides, and low-quality sequences, and the retained sequences were aligned against the mouse genome (mm10).

Predictive causal analysis was performed using the IPA software (Ingenuity, Redwood City, CA, USA). Gene expression profiles were compared among WT-VEH, WT-ATRA, RARα-VEH, and RARα-ATRA groups. Genes with RPKM > 3 were used for the analysis. Up- and down-regulated genes were defined as those showing fold changes of ≥ 2 and ≤ 0.5 respectively. Differentially expressed gene lists were imported into the IPA software and subjected to core analysis. Pathway enrichment was examined through Fisher's exact test.

RNA-seq data have been deposited in the DNA Data Bank of Japan (DDBJ) under BioProject accession number: PRJDB42328. The processed RNA-seq data are also available in Supplementary File 3 (Source Data.xlsx).

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Behavioral experiments

Prior to behavioral experiments, mice were individually handled 2 min per day for 1 week. All experiments were blinded to the experimental group. Individual mice were used for all experiments. Animal behavior was recorded using a video camera.

Social recognition task

The social recognition task was performed as previously described [9, 14, 18]. Briefly, adult mice were placed into individual plastic cages in an experimental room. The cages were identical to the mice' home cages (plastic, 30 cm × 17 cm × 12 cm). After a period of 60 min, a juvenile mouse was placed into a cage with the adult mouse for a training trial that lasted either 1.5 min or 3 min, as indicated in each figure. The duration of social investigation displayed by the adult mouse was quantified using a stopwatch. Social investigation was defined as previously described [9, 14, 18]. Memory for social recognition was assessed in a test session, performed 24 h after training, by quantifying the duration of social investigation of the adult mouse towards the same juvenile.

Group differences in social recognition memory were analyzed using a recognition index calculated as follows: *recognition index = (test investigation time/training investigation time).*

Novel object recognition task

The novel object recognition task was performed as previously described [14]. Briefly, on 3 consecutive days, mice were individually habituated to an open field (40 cm × 40 cm × 40 cm) for 20 min in the

absence of objects. For the training and test sessions, two objects were placed in the open field and the mouse was allowed to explore for 7.5 min. In the test session, performed 24 h after training, mice were re-exposed to one familiar object and exposed to one novel object. In both sessions, the time spent exploring each object was recorded by an observer. Object exploration was defined as occurring when the head of the mouse was facing and within 1–2 cm of the object or when any part of the body, excluding the tail, was physically touching the object. Objects were matched for size, weight, and innate interest levels based on preliminary tests, and were thoroughly cleaned between trials to eliminate olfactory cues. Memory was operationally defined as the proportion of time spent investigating the novel object minus the proportion spent investigating the familiar object.

Group differences were analyzed using a discrimination index, calculated as follows:

Discrimination Index = (novel object exploration time/total exploration time) − (familiar object exploration time/total exploration time)

Contextual fear conditioning task

The contextual fear conditioning task was performed as previously described [17, 18]. Mice were trained and tested in conditioning chambers (17.5 cm × 17.5 cm × 15 cm) fitted with a stainless-steel grid floor through which footshocks could be delivered. Training consisted of placing the mice in the chamber and delivering a footshock (2 s duration) 148 s later. Mice were returned to the home cage 30 s after the shock. The footshock intensity was 0.4 mA for experiments with Ro41 and 0.1 mA for experiments with ATRA or 9-cis RA. Memory was quantified as the percentage of time spent freezing over 5 min, when the mice were placed in the chamber 24 h later. Freezing behavior, defined as the complete lack of movement except for respiration, was measured automatically by the Contextual and Cued Fear Conditioning test system (O'HARA & CO. LTD., Tokyo, Japan).

Reporter assay

Reporter assay was performed as previously described [18, 21]. Briefly, a 1.6 kb fragment of the mouse Chrm4 promoter region (− 1,453 to + 162 bp relative to the transcription start site) was amplified by PCR reaction using mouse tail DNA as a template, using the following primers (5' to 3'):

Forward: GGGGGTACCTTCTGCCCTCTCCCATACAC

Reverse: GGGGAGCTCACGCGTAACTGGAGACAGGTGGGCAG

The amplified PCR fragment was inserted into pBluescript using KpnI and SacI sites, and then subcloned into the pGL3-Basic vector using KpnI and MluI sites. For luciferase assays, NIH-3T3 cells were plated in 24-well plates and transfected 16 h later using a lipofection method. At the time of transfection, the cells were treated with ATRA or 9-cis RA dissolved in DMSO. At 48 h after transfection, the cells were lysed and luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity. Data are presented as mean ± standard deviation (SD). Statistical analyses were

performed using one-way ANOVA followed by post hoc multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

Data analysis

Statistical analysis was performed as previously described, using StatCel 4 (OMS, Japan) [19, 22]. All data are presented as mean ± SEM. Statistical analyses were performed using two-tailed paired or unpaired Student's t tests, one-way ANOVA followed by Dunnett's or Tukey–Kramer post hoc test, or two-way ANOVA followed by Dunnett's or Tukey–Kramer post hoc test, as appropriate. For the social recognition and novel object recognition tasks, paired Student's t test was used for within-group comparisons, whereas unpaired Student's t test or ANOVA followed by the appropriate post hoc test was used for between-group comparisons. A level of $P < 0.05$ was considered statistically significant.

Results

Pharmacological loss- and gain-of-function of hippocampal VA-RA signaling impairs and enhances long-term memory (LTM) formation, respectively

To investigate whether hippocampal VA-RA signaling positively regulates learning and memory, we first examined the effects of pharmacological loss- and gain-of-function in the dorsal hippocampus on hippocampus-dependent memories. For this purpose, Ro41-5253 (Ro41), a selective RARα antagonist, ATRA, a RAR agonist, or 9-cis RA, a RAR and RXR agonist, were microinfused into the dorsal hippocampus, 4 h before training in two hippocampus-dependent tasks: social recognition and contextual fear memory tasks [23, 24] (Fig. 1a).

We first performed the social recognition task [9, 14, 17, 18, 23]. A wild-type (WT) adult mouse was exposed to a same-sex juvenile mouse for 3 (Ro41) or 1.5 (ATRA or 9-cis) min during training, then re-exposed to the same juvenile 24 h later (test), for 3 or 1.5 min, respectively, to examine LTM. Social investigation time during training and test was measured and compared. Memory performance was assessed as the recognition index of social investigation time ratio (test to training; Figs. 1b–d and 1h). We have previously shown that task difficulty can be manipulated through duration of social exposure, where 3 min corresponds to a strong training condition and 1.5 min corresponds to a weak training condition [9, 14, 18].

Under the strong training condition, vehicle-microinfused WT mice (VEH mice) showed significantly decreased social investigation time in test compared with training (paired t-test, $p < 0.05$). In contrast, Ro41-microinfused mice (Ro41 mice) showed comparable social investigation time in training and test ($p > 0.05$), suggesting that VEH mice exhibited social recognition memory, while Ro41-treated mice did not. Consistently, VEH mice showed a significantly lower recognition index compared to Ro41 mice (t-test, $p < 0.05$), indicating that Ro41 mice showed worse memory than VEH mice (Fig. 1b).

In contrast, under the weak training condition, ATRA- or 9-cis RA-microinfused mice (ATRA or 9-cis RA mice) showed a significant decrease in social investigation time during test compared with training (t-

test, $p < 0.05$), whereas this decrease was not observed in VEH mice ($p > 0.05$). This suggests that ATRA- and 9-cis RA mice, but not VEH mice, exhibited social recognition memory. Consistently, ATRA and 9-cis RA mice showed a significantly lower recognition index compared to VEH mice ($P < 0.05$), indicating that ATRA and 9-cis RA mice showed better memory than VEH mice (Figs. 1c and 1d).

Next, we performed the contextual fear conditioning task [17, 18, 24]. Mice were trained with an electrical footshock in a conditioning chamber (training), and 24 h later LTM was tested in the same chamber by assessing freezing behavior as an indicator of contextual fear memory (Test) (Figs. 1e-g). Under the strong training condition and using a 0.4 mA electrical footshock, Ro41-treated mice showed significantly lower freezing than vehicle (VEH)-treated mice in the test session (t-test, $p < 0.05$; Fig. 1e). In contrast, ATRA and 9-cis RA-treated mice showed significantly higher freezing than VEH-treated mice (t-test, both $p < 0.05$; Figs. 1f and 1g). These results suggest that pharmacological loss- and gain-of-function of hippocampal VA-RA signaling impairs and enhances contextual fear memory, respectively.

Our results suggest that pharmacological manipulations of loss- and gain-of-function of hippocampal VA-RA signaling impair and enhance social recognition and contextual fear memories, respectively. To further examine how gain-of-function of VA-RA signaling enhances learning/short-term memory (STM) or LTM formation, we examined its effect on STM both for social recognition and contextual fear (Figs. 1h and 1i). The behavior tests were performed as described above, except that mice were tested 2 h after the training session. VEH and ATRA mice showed comparable recognition index (t-test, $p < 0.05$), with both groups exhibiting a significant decrease in social investigation time during test compared with training (t-test, both $p < 0.05$) (Fig. 1h). Similarly, both groups showed comparable freezing during test (t-test, both $p > 0.05$) in the contextual fear conditioning task (t-test, Fig. 1i). These findings suggest that VEH and ATRA-treated mice exhibit comparable STMs of social recognition and contextual fear conditioning.

Taken together, these results suggest that pharmacological gain-of-function of VA-RA signaling in the hippocampus facilitates hippocampus-dependent LTM formation without affecting learning and STM.

Generation and improved memory performance of RARα mice expressing RARα in the forebrain.

To understand the molecular basis of the positive regulation of hippocampus-dependent LTM by VA-RA signaling, we generated RARα mice that overexpress RARα in the forebrain. For this purpose, we used a tetracycline-dependent transcriptional regulatory system under the forebrain-specific αCaMKII promoter, which induces gain-of-function of VA-RA signaling [9, 14] (Fig. 2a). Northern blot analysis confirmed mRNA expression of the transgene in the forebrain and hippocampus (Figs. S1a and S1b) of the generated mutant mice. Double transgenic (S-09 line × tTA line) mice (RARα mice) were used, as they clearly express the transgene in the forebrain and hippocampus. qRT-PCR analysis revealed that RARα mice showed approximately threefold higher hippocampal RARα mRNA levels than WT mice (Fig. 2b).

Similar to the pharmacological experiments described above, we examined whether RARα mice, with or without intraperitoneal ATRA administration 4 h before training, exhibited enhanced memory. Under the

weak training condition of the social recognition task, RARα mice treated with either VEH or ATRA (VEH- or ATRA-RARα mice) showed significantly decreased social investigation time during test session compared with training (t-test, both $p < 0.05$), whereas WT treated with either VEH or ATRA (VEH- or ATRA-WT mice) did not (t-test, both $p > 0.05$) (Fig. 2c). This suggests that RARα mice exhibit social recognition memory even in the absence of ATRA. Consistently, two-way ANOVA comparing recognition indexes revealed a significant effect of genotype ($F[1,55] = 18.82$, $p < 0.05$). Tukey-Kramer post hoc test showed that ATRA-RARα mice exhibited a significantly better recognition index than WT mice, with or without ATRA injection, whereas VEH-RARα mice did so only compared with VEH-WT mice. These observations suggest that ATRA-RARα mice performed better than other groups, although VEH-RARα and ATRA-RARα mice did not differ significantly. Most importantly, our observations suggest that RARα expression is sufficient to improve memory performance, irrespective of ATRA treatment.

We next performed the novel object recognition task (Fig. 2d). Mice were first exposed to two different objects (A and B) in the open field (training), then 24 h later they were exposed to the familiar object (A or B) and one novel object (C) (test) to examine LTM. For both training and test, the mice were exposed to the objects for 7.5 min, since we previously observed enhanced object recognition memory under this weak training condition [14, 18]. During test VEH- and ATRA-RARα mice, but not VEH- and ATRA-WT mice, spent significantly more time investigating the novel object than the familiar object (Fig. S2a), showing a preference to explore the novel object (t-test, both $p < 0.05$), although all groups showed comparable investigation time and exploration preference for the novel and familiar objects during training (both $p > 0.05$) (Fig. 2d). These observations suggest that RARα mice formed a novel object memory, regardless of ATRA treatment. Consistently, both VEH- and ATRA-RARα mice showed a significantly higher discrimination index than VEH-WT mice (Two-way ANOVA, genotype, $F[1,47] = 8.097$, $p < 0.05$; Tukey-Kramer post hoc test, both $p < 0.05$; Fig. 2b), suggesting that RARα mice exhibit better object recognition memory than WT mice.

Collectively, our observations suggest that genetic gain-of-function of VA-RA signaling, achieved by overexpressing RARα in the forebrain, enhances LTM.

Gene expression regulation by the VA-RA signaling reflected by memory enhancement

We next examined the effects of pharmacological and genetic activation of VA-RA signaling on the expression levels of RAR/RXRs target genes, including RARβ and Cyp26a1 (cytochrome P450, family 26, subfamily a, polypeptide 1), which are widely used indicators of VA-RA signaling activity in the hippocampus [1, 9, 25]. Hippocampal mRNA levels were measured in WT and RARα mice, 4 h after intraperitoneal VEH or ATRA administration, using qRT-PCR analysis (Fig. S3a). Two-way ANOVA revealed a significant effect of drug, and a drug × genotype interaction for RARβ mRNA levels (drug, $F[1, 34] = 65.67$, $p < 0.05$; genotype × drug interaction, $F[1, 34] = 6.11$, $p < 0.05$), and significant effects of drug and genotype for Cyp26a1 mRNA levels (genotype, $F[1,40] = 12.04$, $p < 0.05$; drug, $F[1,40] = 9.67$, $p < 0.05$). RARβ and Cyp26a1 mRNA levels were significantly higher in the hippocampus of ATRA-WT and ATRA-

RARα mice compared to the VEH-WT and ATRA-RARα mice (ps > 0.05), respectively. Consistent with previous findings, these observations suggest that induction of mRNA expression depends on the ATRA treatment. ATRA-RARα mice showed higher (but not statistically significant) RARβ and Cyp26a1 mRNA expressions than ATRA WT mice, suggesting that RARα overexpression itself may contribute for increased Cyp26a1 and RARβ mRNA expression.

To understand the molecular basis of VA-RA signaling-related memory enhancement, we performed bulk RNA-seq of the hippocampus of WT and RARα mice, 4 h after intraperitoneal injection of VEH or ATRA, and compared transcription profiles among VEH-WT, ATRA-WT, VEH-RARα, and ATRA-RARα mice (Figs. 3a, S3b).

Compared with VEH-WT mice, ATRA-WT, VEH-RARα, and ATRA-RARα mice showed significantly increased mRNA expression of 834, 998, and 1025 genes, respectively, and decreased mRNA expression of 815, 1131, and 1056 genes, respectively, in the hippocampus. Importantly, among mRNAs with increased expression, 778 were common to ATRA-WT, VEH-RARα, and ATRA-RARα mice, whereas among mRNAs showing decreased expression, 760 were common to the three mouse groups (Fig. 3b). These observations indicate that VEH-RARα mice exhibited similar transcription profiles to ATRA-WT mice, suggesting that overexpression of RARα is sufficient to induce similar mRNA expression profiles to those achieved with ATRA treatment.

We next performed Gene Ontology analyses using IPA to compare expression profiles between VEH-WT and ATRA-RARα mice (Fig. 3c). IPA revealed 4 main pathways regulated by VA-RA signaling activation: hepatic fibrosis, axonal guidance signaling, cAMP-mediated signaling, and G-protein coupled receptor signaling (Fig. 3c).

To validate RNA-seq and IPA, we used qRT-PCR to measure the mRNA levels of cholinergic receptor muscarinic 4 (Chrm4), AKT serine/threonine kinase 3 (Akt3), adenylate cyclase 1 (Adcy1), and cannabinoid receptor 1 (Cnr1), since these genes are involved in memory processes [26–29]. Two-way ANOVA revealed a significant effect of drug on Chrm4, Akt3, and CB1 mRNA levels, and a significant effect of genotype on Adcy1 mRNA levels (Fig. 3d). Subsequent Dunnett post hoc test showed that ATRA-WT and ATRA-RARα mice showed significantly higher hippocampal Akt3 mRNA expression than VEH-WT mice ($p < 0.05$), suggesting that this increase largely depends on ATRA treatment. ATRA-RARα mice showed significantly higher hippocampal expression levels of Chrm4 and CB1 mRNA compared to VEH-WT mice (ps < 0.05), but not ATRA-WT and VEH- RARα mice (ps > 0.05), suggesting that both ATRA treatment and RARα overexpression are required for increased expression of these genes. Interestingly, VEH- and ATRA-RARα mice showed significantly higher hippocampal Adcy1 mRNA expression than VEH and ATRA WT mice ($p < 0.05$), suggesting that this increase depends on RARα overexpression. These observations suggest that transcription of target genes is differently regulated by ATRA treatment and RARα overexpression.

We next investigated the molecular mechanism of memory enhancement by activating VA-RA signaling. For this purpose, we investigated the roles of Chrm4 in social recognition and novel object memory.

Social recognition task was performed under a weak training condition, as described above, except that mice received an intraperitoneal injection of OXO, a muscarinic acetylcholine receptor agonist, 10 minutes after training (Fig. 3e). OXO- but not VEH-treated mice showed significantly decreased social investigation time in test compared with training (t-test, $p < 0.05$), suggesting that OXO-treated mice exhibited social recognition memory while VEH mice did not (Fig. 3e). Consistently, OXO-treated mice showed a significantly better recognition index compared to VEH mice (t-test, $p < 0.05$), indicating that OXO treatment enhances social recognition memory (Fig. 3e).

We next performed the novel object memory task under a weak training condition (7.5 min exposure) (Fig. 3f). Consistent with the results of social recognition, OXO- but not VEH-treated WT mice spent significantly longer investigating the novel object than the familiar object (Fig. S4a), exhibiting an exploration preference for the novel object (t-test, $p < 0.05$) (Fig. 3f), although both groups showed comparable performance during training (t-test, $p > 0.05$). This suggests that OXO-treated WT mice exhibited object memory. OXO-treated WT mice also showed a significantly higher discrimination index than VEH-treated WT mice (Fig. 3f), suggesting that OXO treatment enhances object memory. Collectively, these observations suggest that Chrm4 activation improves memory performance.

Our findings raised the hypothesis that VA-RA signaling positively regulates the activity of the Chrm4 promoter. To investigate this hypothesis, we finally examined the effects of VA-RA signaling activation on Chrm4 promoter activity in NIH3T3 cells using a luciferase assay (Fig. 3g). The cells were transiently transfected with the luciferase reporter construct harboring approximately 1.6 kb of the *Chrm4* promoter region (− 1,453 to + 162 bp relative to the transcription start site) or the corresponding basic vector and then were treated with ATRA or 9-cis RA for 48 h. ATRA or 9-cis RA treatment significantly increased luciferase activity in a dose-dependent manner when the reporter contained the Chrm4 promoter sequence. These observations suggest that VA-RA signaling increases Chrm4 expression by activating the promoter, thereby contributing to memory enhancement.

Discussion

Previous studies have shown that loss-of-function in the VA-RA signaling pathway, associated with VA deficiency or deletion of RAR/RXR genes, impairs synaptic plasticity and memory [5, 7, 10–13]. However, the effects of gain-of-function of VA-RA signaling remain unknown. In the present study, we found that pharmacological gain-of-function of the VA-RA signaling pathway enhanced hippocampus-dependent LTM without affecting STM, while loss of VA-RA function impairs this memory. Interestingly, genetic gain-of-function of this pathway, achieved by overexpressing RARα in the forebrain, also enhanced hippocampus-dependent LTM, even without ATRA treatment. Additionally, we found that activating the VA-RA signaling pathway by ATRA injection and/or RARα overexpression altered hippocampal expression of numerous mRNAs, including those involved in axonal guidance signaling, cAMP-mediated signaling, and G-protein-coupled receptor signaling, which play critical roles in synaptic plasticity and learning and memory. These findings suggest that VA-RA signaling activation enhances memory formation by altering transcription implicated in hippocampus-dependent learning and memory.

Interestingly, we observed that RARα mice showed significantly better memory performance than WT mice, even in the absence of ATRA treatment. This suggests that RARα overexpression is sufficient to improve memory performance in the absence of RA. Similarly, RNA-seq analysis indicated that RARα overexpression exerted a similar effect on transcription profiles to ATRA treatment. Therefore, it is possible that RARα overexpression increases sensitivity to endogenous RAs, thereby altering transcription profiles similarly to ATRA induction, and enhancing memory. Interestingly, we found that RARα mice showed increased Adcy1 mRNA levels in the hippocampus irrespective of ATRA treatment. Adcy1 activates the cAMP-signaling pathway by synthesizing cAMP, leading to PKA-mediated phosphorylation of the transcription factor CREB at Serine 133 and the AMPA glutamate receptor GluA1 subunit at Serine 845. Our previous studies have shown that PKA facilitation of CREB or GluA1 phosphorylation in the forebrain enhances memory formation and retrieval, respectively [14, 18]. Therefore, the memory-enhancing effects of RARα overexpression may be mediated by increased Adcy1 expression, which activates CREB and AMPA receptors.

Interestingly, loss-of-function of VA-RA signaling reduces striatal Chrm4 expression [30]. In the present study, we found that systemic ATRA administration significantly increased hippocampal Chrm4 mRNA levels, and both ATRA and 9-cis RA activate transcription from the Chrm4 promoter. Taken together, these findings suggest that the Chrm4 gene is a target of the VA-RA signaling pathway, although further analysis of the Chrm4 promoter is required. Furthermore, systemic administration of a Chrm4 agonist enhanced social and novel-object memories, which aligns with previous studies showing that Chrm4 plays a critical role in learning and memory [31]. Therefore, these findings raise the possibility that the memory-enhancing effects of the VA-RA signaling pathway are mediated by its upregulation of Chrm4.

Hippocampus-dependent LTM was enhanced by VA-RA signaling activation through intrahippocampal microinfusion of RAR/RXR agonists. In contrast, systemic ATRA administration did not significantly enhance memory under the present experimental conditions, although ATRA-treated WT and RARα mice showed slightly, but not significantly, better indexes of social recognition and novel object memories compared to the respective untreated groups. Therefore, it is possible that memory enhancement requires microinfusing a higher dose of agonists in the brain compared to systemic injection, although lower agonist doses in the brain achieved by systemic injection are sufficient to induce transcriptional changes. Another possibility is that systemic ATRA treatment may require repeated administration over several days or weeks, to achieve memory-enhancing effects. This possibility is supported by previous studies showing beneficial effects of systemic retinoid treatment across repeated treatment schedules in aged or pathological animal models [10–12].

Similarly, systemic ATRA treatment was sufficient to up- and down-regulate numerous mRNA levels in the hippocampus, although it failed to improve memory. In contrast, RARα overexpression alone improved memory, although it showed a less pronounced increase in mRNA levels than ATRA treatment, when measured by qRT-PCR. In the present study mRNA levels were only quantified at one time point, 4 h after ATRA treatment. Therefore, it is possible that RARα overexpression increased the expression of memory-enhancing proteins weakly but prolongedly, whereas ATRA treatment did so strongly but

transiently. Future studies are required to analyze mRNA levels at multiple time points and compare the effects of RARα overexpression and ATRA treatment on transcription profiles and memory performance.

RARα is present in the hippocampal dendrites and induces the translation of GluA1 in a RA-dependent manner, but independently of transcriptional regulation, resulting in the increased membrane insertion of GluA1 [32–35]. This increased translation and membrane translocation of GluA1 is thought to contribute to improved memory performance and long-term neural plasticity. Therefore, it is possible that RARα overexpression facilitates GluA1 translation in a transcription-independent manner, thereby improving memory. This may be why RARα overexpression fails to show a pronounced increase in mRNAs but causes memory enhancement.

The present findings should be carefully interpreted, since VA-RA signaling plays important roles both in development and differentiation and in adult brain function [1, 3, 4]. Furthermore, RARα overexpression in the forebrain may affect brain development, whereas systemic manipulation of the VA-RA signaling pathway may affect multiple tissues and biological processes outside the hippocampus. Further studies are required to determine the treatment conditions, such as inducible expression of RARα only in the hippocampus, that can selectively enhance memory-related function while minimizing broader systemic effects.

In summary, we found that pharmacological or genetic gain-of-function of the VA-RA signaling pathway, induced by ATRA treatment or RARα overexpression, respectively, enhances hippocampus-dependent memory. We also showed that ATRA treatment and/or RARα overexpression alter hippocampal transcription profiles and that the Chrm4 gene is a VA-RA signaling pathway target that enhances memory, suggesting an underlying molecular mechanism of VA-RA-mediated memory enhancement. Thus, our findings suggest that the nutrient factors VA and RA function as memory enhancers.

Abbreviations

RA, retinoic acid;

ATRA, all-trans retinoic acid,

RAR, retinoic acid receptor;

RXR, retinoid X receptor;

RNA-seq, RNA-sequencing;

ANOVA, analysis of variance/

Declarations

Competing interests

The authors declare no competing interests.

Authors' information

Not applicable.

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Author Contribution

SK is responsible for the hypothesis development and overall design of the research and experiment, and supervised the experimental analyses. SK and MN co-wrote the manuscript. MN, SY, YT, TS and TE performed behavioral analyses. MN, TE and SH performed biochemical analyses. MN and SU constructed a transgene and generated transgenic mice expressing RARα. ST and DM performed luciferase assay. All authors read and approved this manuscript.

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Figures

Figure 1

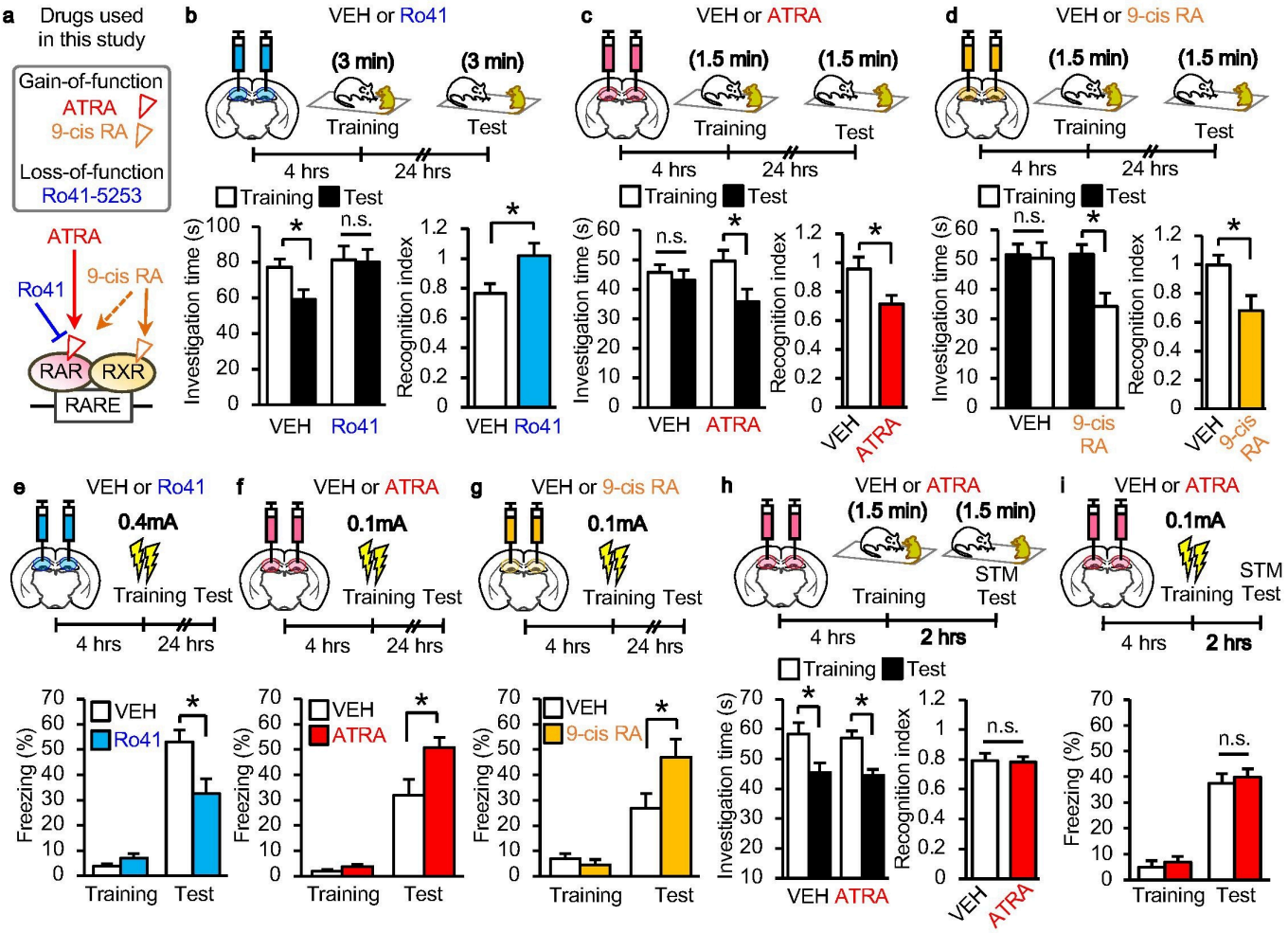


Figure 1

Pharmacological gain- and loss-of-function of hippocampal VA-RA signaling bidirectionally regulate long-term memory

a Schematic illustration of pharmacological loss- and gain-of-function of VA-RA signaling using Ro41-5253 (Ro41), all-trans retinoic acid (ATRA), and 9-cis retinoic acid (9-cis RA).

b–d, Social recognition task. **b** Social recognition task under strong training condition (3 min).. Ro41 impaired long-term social recognition memory tested 24 h later. (VEH, *n* = 10; Ro41, *n* = 10). Two-tailed paired Student's t-test for VEH ($p < 0.05$) and Ro41 ($p > 0.05$). Two-tailed unpaired Student's t-test ($p < 0.05$). **c, d** Social recognition task under weak training condition (1.5 min). **c** ATRA enhanced long-term

social recognition memory. (VEH, *n* = 11; Ro41, *n* = 11). Two-tailed paired Student's t-test for VEH ($p > 0.05$) and ATRA ($p < 0.05$). Two-tailed unpaired Student's t-test ($p < 0.05$). **d** 9-cis RA enhanced long-term social recognition memory. (VEH, *n* = 10; 9-cis RA, *n* = 10). Two-tailed paired Student's t-test for VEH ($p > 0.05$) and 9-cis RA ($p < 0.05$). Two-tailed unpaired Student's t-test ($p < 0.05$)

e–g, Contextual fear conditioning. Drugs were microinfused into the dorsal hippocampus 4 h before training under the 0.4 mA foot shock condition, and long-term memory was tested 24 h later. **e** Ro41 impaired long-term contextual fear memory under the 0.1 mA foot shock condition (VEH, *n*= 12; Ro41, *n* = 13). Two-tailed unpaired Student's t-test ($p < 0.05$). **f** ATRA enhanced long-term contextual fear memory under the 0.1 mA foot shock condition. (VEH, *n*= 13; ATRA, *n* = 12). Two-tailed unpaired Student's t-test ($p < 0.05$). **g** 9-cis RA enhanced long-term contextual fear memory. (VEH, *n* = 10; 9-cis RA, *n* = 11). Two-tailed unpaired Student's t-test ($p < 0.05$). **h, i** Short-term memory (STM) tested 2 h after training. **h** ATRA did not affect short-term social recognition memory. (VEH, *n* = 10; ATRA, *n* = 9). Two-tailed paired Student's t-test for VEH ($p < 0.05$) and ATRA ($p < 0.05$). Two-tailed unpaired Student's t-test ($p > 0.05$). **i** ATRA did not affect short-term contextual fear memory under the 0.1 mA foot shock condition. (VEH, *n* = 18; ATRA, *n* = 16). Two-tailed unpaired Student's t-test ($p > 0.05$) Data are shown as mean ± SEM. *P < 0.05; n.s., not significant; VEH, vehicle; ATRA, all-trans retinoic acid.

Figure 2

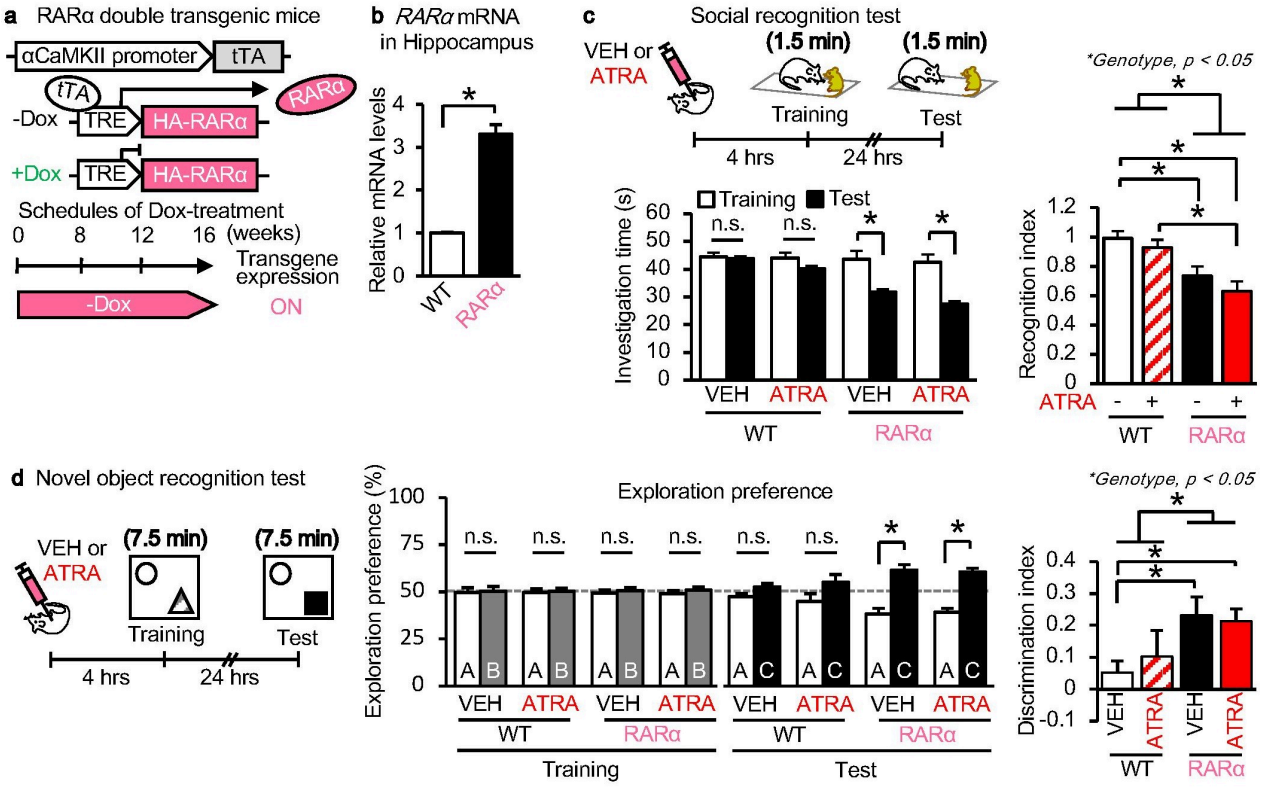


Figure 2

Generation of RARα transgenic mice expressing RARα in the forebrain and enhanced LTM by genetic gain-of-function of VA-RA signaling.

a Schematic illustration of the generation of RARα double transgenic mice using the tetracycline-dependent transcriptional regulatory system under the forebrain-specific αCaMKII promoter. **b** Hippocampal RARα mRNA levels in WT and RARα mice. Two-tailed unpaired Student's t-test ($p< 0.05$). **c** Social recognition task under weak training condition (1.5 min). RARα mice showed enhanced long-term social recognition memory. Two-tailed paired Student's t-test was used to analyze investigation time. Significant differences are indicated by asterisks (*p < 0.05) or n.s. (p > 0.05). Two-way ANOVA followed by Tukey-Kramer post hoc test. For recognition index (WT-VEH, $n= 20$; WT-ATRA, $n = 19$; RAR-VEH, $n = 9$; RAR-ATRA, $n = 8$; genotype, $F[1,55] = 18.82$, $p < 0.05$). Significant post hoc comparisons are indicated by asterisks in the graph. **d** Novel object recognition task under weak training condition (7.5 min). RARα mice showed enhanced long-term object recognition memory. Two-tailed paired Student's t-test was used to analyze investigation time. Significant differences are indicated by asterisks (*p < 0.05) or n.s. (p > 0.05). Two-way ANOVA followed by Tukey-Kramer post hoc test. For discrimination index (WT-VEH, $n = 12$; WT-ATRA, $n = 9$; RAR-VEH, $n = 12$; RAR-ATRA, $n= 15$; genotype, $F[1,47] = 8.097$, $p < 0.05$). Significant post hoc comparisons are indicated by asterisks in the graph. Data are shown as mean ± SEM. *$p < 0.05$; n.s., not significant; VEH, vehicle; ATRA, all-trans retinoic acid.

Figure 3

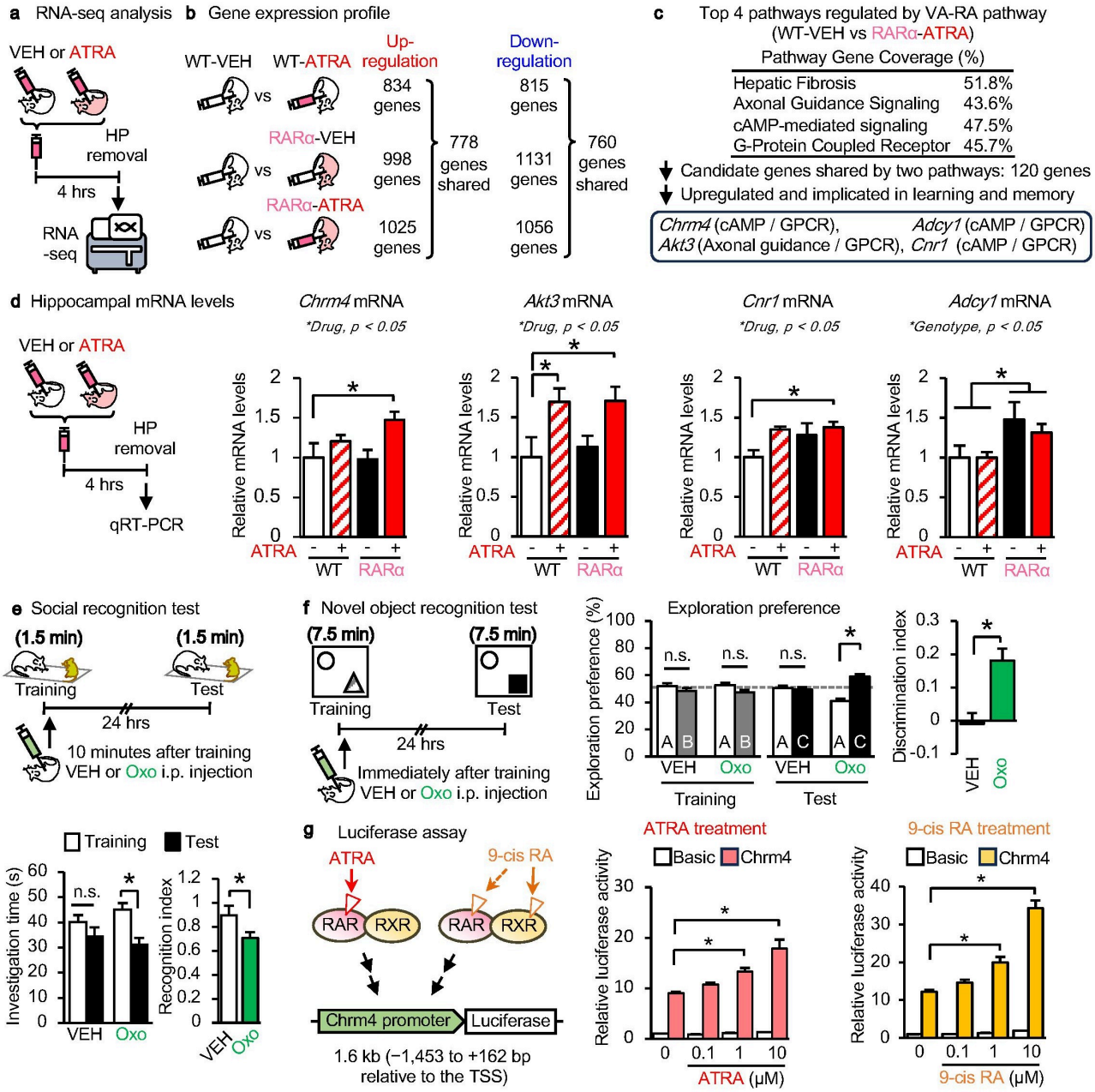


Figure 3

Transcriptional regulation by gain-of-function of VA-RA signaling, and roles of Chrm4 in long-term memory and the regulation of its promoter activity by VA-RA signaling

a Experimental design for RNA-seq analysis of the hippocampus 4 h after intraperitoneal injection of VEH or ATRA. **b** Summary of differentially expressed genes identified in WT-ATRA, RAR-VEH, and RAR-

ATRA mice relative to WT-VEH mice. The numbers of shared gene sets of upregulated and downregulated genes are indicated. **f** Pathway analysis of genes altered in the comparison between WT-VEH and RAR-ATRA. Top 4 pathways regulated by activation of the VA-RA signaling and candidate selected genes associated with learning and memory are shown. **c** qRT-PCR analysis of candidate genes in the hippocampus. Chrm4, Akt3, and Cnr1 were responsive mainly to ATRA treatment, whereas Adcy1 was associated mainly with RARα overexpression. Two-way ANOVA followed by Dunnett post hoc test. For *Chrm4* (WT-VEH, n = 5; WT-ATRA, n = 8; RAR-VEH, n = 6; RAR-ATRA, n = 6; drug, $F[1,24] = 8.792$, $p < 0.05$). For *Akt3* (WT-VEH, n = 5; WT-ATRA, n= 8; RAR-VEH, n = 6; RAR-ATRA, n = 6; drug, $F[1,24] = 11.50$, $p < 0.05$). For *Cnr1* (WT-VEH, n = 7; WT-ATRA, n = 6; RAR-VEH, n = 6; RAR-ATRA, n = 6; drug, $F[1,24] = 8.792$, $p < 0.05$). For *Adcy1* (WT-VEH, n = 6; WT-ATRA, n= 6; RAR-VEH, n = 6; RAR-ATRA, n = 6; genotype, $F[1,23] = 7.226$, $p < 0.05$). Significant post hoc comparisons are indicated by asterisks in the graph. Data are shown as mean ± SEM. *$p < 0.05$; n.s., not significant; VEH, vehicle; ATRA, all-trans retinoic acid.

e, f Effect of Oxotremorine (OXO) on memory formation. **e** Social recognition task under weak training condition (1.5 min). OXO-treated mice showed enhanced long-term social recognition memory. (VEH, n = 15; OXO, n = 18). Two-tailed paired Student's t-test for investigation time. VEH ($p > 0.05$) and OXO ($p< 0.05$). Two-tailed unpaired Student's t-test for recognition index ($p< 0.05$). **f** Intraperitoneal injection of Oxotremorine (OXO) immediately after training enhanced long-term novel object recognition memory. (VEH, n = 9; OXO, n = 14). Two-tailed paired Student's t-test was used to analyze investigation time and exploration preference. Significant differences are indicated by asterisks (*p < 0.05) or n.s. (p > 0.05). Two-tailed unpaired Student's t-test for discrimination index. ($p < 0.05$) **g** Schematic diagram of luciferase assay for effect of ATRA and 9-cis RA on Chrm4 promoter activity. Luciferase assay showing that ATRA and 9-cis RA increased Chrm4 promoter activity in a dose-dependent manner. One-way ANOVA followed by Dunnett post hoc test. For 9-cis RA (each dose, n = 3; $F[3,11] = 58.19$, $p< 0.05$). For ATRA (each dose, n = 3; $F[3,11] = 14.57$, $p< 0.05$). Significant post hoc comparisons are indicated by asterisks in the graph. Data are shown as mean ± SEM. *$p< 0.05$; n.s., not significant; VEH, vehicle; ATRA, all-trans retinoic acid; kb, kilo base pairs. TSS, transcription start site.