RGS14$_{414}$ treatment induces memory enhancement and rescues episodic memory deficits

Mariam Masmudi-Martín,[*,†,1] Irene Navarro-Lobato,[*,†,1] Manuel F. López-Aranda,[*,†,2] Gloria Delgado,[*,†] Elisa Martín-Montañez,[‡,§] Maria E. Quiros-Ortega,[*,†] Marta Carretero-Rey,[*,†] Lucía Narváez,[*,†] Maria F. Garcia-Garrido,[*,†,3] Sinforiano Posadas,[*,†] Juan F. López-Téllez,[*,†] Eduardo Blanco,[¶] Inmaculada Jiménez-Recuerda,[*,†] Pablo Granados-Durán,[*,†] Jose Paez-Rueda,[*] Juan C. López, and Zafar U. Khan[*,†,§,#,4]

*Laboratory of Neurobiology, Centro de Investigaciones Medico Sanitaria (CIMES), †Department of Medicine, and ‡Department of Pharmacology, Faculty of Medicine, Campus Teatinos, and §Institute of Biomedical Research in Malaga (IBIMA), University of Malaga, Malaga, Spain; ¶ Department of Pedagogy and Psychology, University of Lleida, Lleida, Spain; Animal Behavior and Neuroscience Laboratory, Department of Experimental Psychology, Faculty of Psychology, University of Seville, Seville, Spain; and #Centro Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED), Institute of Health Carlos III, Madrid, Spain

ABSTRACT:

Memory deficits affect a large proportion of the human population and are associated with aging and many neurologic, neurodegenerative, and psychiatric diseases. Treatment of this mental disorder has been disappointing because all potential candidates studied thus far have failed to produce consistent effects across various types of memory and have shown limited to no effects on memory deficits. Here, we show that the promotion of neuronal arborization through the expression of the regulator of G-protein signaling 14 of 414 amino acids (RGS14$_{414}$) not only induced robust enhancement of multiple types of memory but was also sufficient for the recovery of recognition, spatial, and temporal memory, which are kinds of episodic memory that are primarily affected in patients or individuals with memory dysfunction. We observed that a surge in neuronal arborization was mediated by up-regulation of brain-derived neurotrophic factor (BDNF) signaling and that the deletion of BDNF abrogated both neuronal arborization activation and memory enhancement. The activation of BDNF-dependent neuronal arborization generated almost 2-fold increases in synapse numbers in dendrites of pyramidal neurons and in neurites of nonpyramidal neurons. This increase in synaptic connections might have evoked reorganization within neuronal circuits and eventually supported an increase in the activity of such circuits. Thus, in addition to showing the potential of RGS14414 for rescuing memory deficits, our results suggest that a boost in circuit activity could facilitate memory enhancement and the reversal of memory deficits.

KEY WORDS: cognitive dysfunction, BDNF, memory circuit activation, recovery of memory functions behavioral performance.

ABBREVIATIONS: AD, Alzheimer disease; AP, anteroposterior; BDNF, brain-derived neurotrophic factor; CA1, cornu ammonis 1; CC, choice compartment; CREB, cAMP response element binding; CTA, conditioned taste aversion; DI, discrimination index; DV, dorsoventral; FGF, fibroblast growth factor; LiCl, lithium chloride; ML, mediolateral; MWM, Morris water maze; NGF, nerve growth factor; ORM, object recognition memory; Ox7-SAP, thy 1-saporin; PRh, perirhinal cortex; qRT-PCR, quantitative RT-PCR; RGS14414, regulator of G-protein signaling 14 of 414 amino acids; TrKb, tropomyosin receptor kinase B

1 These authors contributed equally to this work.

2 Current affiliation: Gonda Neuroscience and Genetics Center, University of California–Los Angeles, Los Angeles, CA, USA.

3 Current affiliation: Hatherly Laboratories, University of Exeter Medical School, Exeter, United Kingdom.

4 Correspondence: Zafar U. Khan; e-mail: zkhan@uma.es

Intact memory function is critical for carrying out daily life activities, such as managing finances, remembering to take medications, driving in familiar environments, remembering a grandchild's birthday, and learning to use a new computer. However, memory deficits not only accompany normal aging but are also comorbid with many psychiatric, neurologic, and neurodegenerative diseases Intellectual disability, autism, attention deficit disorder, learning disability, schizophrenia, and depression all have memory deficit components, as do Alzheimer disease (AD), Parkinson disease, Huntington disease, and other neurodegenerative diseases (1–3). Thus, memory deficits affect a large proportion of the human population. Cognition-enhancing pharmacological agents are viewed as a strategy for treating memory deficits or slowing the effects of aging on memory function (4). Some of the most thoroughly studied examples of memory enhancers are partial agonists of the N-methyl-D-aspartate (NMDA) receptor, D-cycloserine and D-serine (5, 6); synthetic ampakines that allow glutamate to exert a prolonged effect on a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (7); stimulant drugs that inhibit monoamine reuptake, including amphetamines (Adderall), methylphenidate (Ritalin), and modafinil (Provigil) (8); and donepezil (Aricept), which was designed to inhibit the enzyme acetylcholinesterase, which is responsible for degrading acetylcholine (9). These agents and other memory enhancers that have been studied to date have failed to produce consistent and invariable effects across various types of memory and have shown limited to no effect on memory deficits (10).

The regulator of G-protein signaling 14 of 414 amino acids ($RGS14_{414}$) gene is a 1245 base pair splice variant (GenBank AY987041) cloned from the human cortex that encodes a protein of 414 aa and is expressed throughout the whole brain (11). In contrast to the complete human (GenBank, NP_006471.2) and rat (GenBank, NC_005116.4) genes, RGS14414 contains a deletion of 153 aa at the N terminus within the RGS domain. This deletion eliminates GTPase activity, a process that is mediated through the RGS domain. The GTPase activity of the RGS domain is thought to be involved in the suppression of

intracellular signaling. Accordingly, studies have shown that complete RGS14 (with the RGS domain) functions as a suppressor of neuronal plasticity and hippocampal-based learning and memory (12, 13). Thus, omission of the RGS domain (as in $RGS14_{414}$) might eliminate this suppressive effect and further aid in memory formation. The facilitation of hippocampal memory processing after the deletion of complete RGS14 from hippocampal CA2 neurons (12) further supports this concept. Although $RGS14_{414}$ lacks a functional RGS domain, it contains a number of other domains, including a G-protein regulatory motif (also known as GoLoco) and 2 Raf-like Ras binding domains (14, 15). Therefore, $RGS14_{414}$ might be involved in other unknown brain functions beyond those requiring GTP hydrolysis. In this study, we explored the role of $RGS14_{414}$ in memory enhancement and in recuperation of memory deficits. Our results demonstrate that $RGS14_{414}$ not only induced the enhancement of multiple types of memory but also promoted the recovery of object, spatial, and temporal memory in memory-deficient rodent models of aging and AD.

MATERIALS AND METHODS

Lentivirus preparation

A cDNA of human RGS14 of 1245 base pair (AY987041; Gen-Bank; https://www.ncbi.nlm.nih.gov/genbank/) that encodes a protein of 414 aa (043566-5; UniProt; https://www.uniprot.org/) was cloned into the pLenti6/Ubc/V5-DEST Gateway vector, and RGS14 lentivirus was produced according to the protocols of the ViraPower Lentiviral Expression System (Thermo Fisher Scientific, Waltham, MA, USA). Vehicle lentivirus (vehicle) was prepared by vector alone.

Animals

In this study, we used rats, transgenic *hAPPSwInd* mouse model of AD, and brain-derived neurotrophic factor (BDNF) knockout mice. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Malaga. Both rats and mice were housed in a temperature-regulated (20 6 2°C) room on a 12-h light/dark cycle. Drinking water and food were available ad libitum except when indicated. The animals were acclimatized to the room for at least 1 wk before starting the experiments, which took place during the light phase.

Rats

Wistar Han rats were used for this study, and they were obtained from Charles River Laboratories (Wilmington, MA, USA).

Transgenic mice with AD

The transgenic hAPPSwInd (J20) mice used in this study were from the The Jackson Laboratory (006293; BarHarbor,ME,USA). These mice express a mutant form of human amyloid precursor protein (APP) bearing both Swedish (K670N/M671L) and Indiana (V717F) mutations associated with familial AD (16). Mutated APP expression is directed

to neurons under the control of the human platelet–derived growth factor b chain promoter. These mice were inbred on the C57BL/6J genetic background. The appearance of Ab deposition has been observed at the age of 6 mo. These mice show synaptic and cognitive impairments, among other characteristics of AD, and have been widely used for cognitive studies (17, 18). Wild-type C57BL/6J mice were used as controls. Four to five mice were housed in each cage.

BDNF knockout mice

BDNF mice were from the The Jackson Laboratory (002266). In our studies, mice heterozygous to BDNF mutation were used, and wild-type C57BL/6 mice were used as controls.

Lentivirus delivery

Rats and mice were anesthetized with the sevoflurane system (induction at 5% sevoflurane +1 L/min O_2 and maintenance at 2% sevoflurane + 0.4 L/minO_2) and placed in a stereotaxic frame according to the coordinates obtained from Paxinos and Watson (19) and Paxinos and Franklin (20). The coordinates of the injection site in the perirhinal cortex (PRh) were anteroposterior (AP) -4.52, mediolateral (ML) ±6.7, and dorsoventral (DV) -4.75 (rats) and AP -1.9, ML ±3.9, and DV -2.20 (mice). The coordinates of the injection site in the rat cornu ammonis 1 (CA1) area were AP-4.3, ML ±3.2, and DV-2.3, and the coordinates of the rat prelimbic cortex were AP +3.7, ML±0.6, and DV-2.9. A total volume of 2 ml of$RGS14_{414}$ lentivirus from a stock titer of 2.1 x 10^7 TU/ml was injected bilaterally through a 30-gauge stainless steel internal cannula. Behavioral tests were performed 3 wk after injection when $RGS14_{414}$ protein expression was observed to be optimal (21).

Object recognition memory test in rats

The object recognition memory (ORM) test was performed as previously described in refs. 21 and 22. Prior to the test, the rats were handled for 8 min daily for 5 consecutive days, and the next 2 d, they were habituated in an open field (100 x 100 x 50 cm) for 12 min. On the day of the experiment and during the exploration session, the rats were placed in the same open field with 2 identical objects and were allowed to explore freely for 3 min. After delays of 15, 30, 45, 60 min, or 24 h, the animals were tested for ORM status with 1 previously presented object and a new object. The exploration time was recorded during exploration and ORM test sessions for both vehicle control–treated and $RGS14_{414}$-treated animals. The average total exploration time of vehicle and RGS14 lentivirus–treated animals was 29.17±1.79 and 28.0± 3.20 s, respectively. Animals who were able to retain the object information during the delay period explored the novel objects for more than 66% of the total exploration time. The discrimination index (DI) was calculated by dividing the time spent exploring the novel object by the total exploration time (novel object + old object). A DI ≤0.5 indicated that the animals were unable to retain object information in memory because they explored both old and new objects equal times (old object 50% and new object 50%), whereas a DI >0.66 was considered to indicate that the animals were able to successfully retain information about the object in memory because

animals spent exploring new object >66% of the total time and <34% of the time exploring old object.

Immunohistochemistry

Immunohistochemistry was performed as previously described in refs. 21–23. In brief, after termination of the behavioral tests, the rats were perfused transcardially with a fixative containing 4% paraformaldehyde, 1.37% L-lysine, and 0.21% meta-periodate and cryoprotected with 30% sucrose. Coronal and sagittal brain sections with a thickness of 30 mm were incubated overnight at 4°C with an affinity purified rabbit anti-RGS14 antibody (1:30 dilution) that was prepared in our laboratory (11). The sections were then incubated for 90 min with Alexa Fluor 568 goat anti-rabbit IgG (1:1000 dilution, A11011; Thermo Fisher Scientific), and immunofluorescence labeling was detected by confocal microscopy.

Cresyl violet staining

Brain sections of rats mounted on gelatin-coated slides were stained for 15 min with 1% cresyl violet. The sections were dehydrated and mounted for analysis under a microscope.

Ox7-SAP injection

The injection of thy 1-saporin (Ox7-SAP; 0.9 mg in 1 ml, IT-02; Advanced Targeting Systems, San Diego, CA, USA) into the PRh was performed in a manner similar to that described in the lentivirus delivery section, using the indicated coordinates. The extent of damage in the brains of these animals was analyzed after staining the brain sections with cresyl violet.

Golgi staining

Rat and mouse brains were processed for Golgi-Cox staining using a Rapid GolgiStain Kit (FD Neurotechnologies, Columbia, MD, USA) following the protocol from the manufacturer. All procedures were performed protected from light. A 180-µm section was prepared with a cryostat, and after staining, the sections were mounted with Permount mounting medium (Thermo Fisher Scientific). Both pyramidal and nonpyramidal neuronal branching were analyzed in the area of injection under a DM IRE2 microscope (Leica Microsystems, Buffalo Grove, IL, USA) using Leica MMAF software, v.1.6.0 (Leica Microsystems). A total of 55–56 pyramidal and nonpyramidal neurons were studied in the vehicle lentivirus–treated group, and 56–79 neurons were studied in the RGS lentivirus–treated group. The number of neurites and the amount of neurite branching in both pyramidal and nonpyramidal neurons and the branching of apical dendrites in pyramidal neurons were determined. Spines were counted from apical dendrites of pyramidal neurons and classified according to their physical appearance into the thin, mushroom, and stubby groups, as previously described by Morrison and Baxter (24).

Quantitative RT-PCR

Brain tissues were processed for RNA extraction with the RNeasy Tissue Mini Kit (Qiagen, Germantown, MD, USA). cDNAs of purified RNA samples were prepared with the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). For quantitative RT-PCR (qRT-PCR), the following specific forward and reverse primers were used: 5´-AAGCAATATTTCTACGAGACCAAGTG-3´(forward), 5´-TACGATTGGGTAGTTCGGCATT-3´ (reverse) for BDNF (NM_001270630.1); 5´-GACGGCTGCTGGCTTCTAAGT-3´ (forward), 5´-TCCGTGACCGGTAAGTGTTG-3´ (reverse) for fibroblast growth factor (FGF; NM_019305.2); and 5´-GCAGACCCGCAACATCACT-3´(forward), 5´-GGTGGAGGCTGGGTGCTAA-3´ (R) for nerve growth factor (NGF; NM_001277055.1). Ribosomal protein L19 (Rpl19) was used to normalize the values. Relative expression values were calculated by a standard curve.

Western blots

Blots were performed as previously described by Khan et al. (25). A4-mmpunchwas extracted from the injected area of the rat brain and homogenized. After treatment with SDS buffer, whole homogenate proteins were separated on 4–20%Mini-ProteinTGX Precast Protein Gels (Bio-Rad, Hercules, CA, USA) and transblotted onto PVDF membranes. Membranes containing proteins were incubated with an anti-BDNF antibody (1:250, sc33904; Santa Cruz Biotechnology, Dallas, TX, USA), followed by incubation with anti-goat IgG-HRP (1:2000; GE Healthcare Life Sciences, Marlborough, MA, USA). Bands were visualized by using an ECL kit (GE Healthcare Life Sciences). A single immunoreactive band of pro-BDNF was identified at 32 kDa. Densitometry analysis of the bands was performed using Quantity One (Bio-Rad), and all values were normalized to a-tubulin.

ORM test in BDNF mice

Except for the exploration session, the ORM test in mice was very similar to that in rats, with the mice being exposed to identical objects for 5min.The size of the open field was smaller (50 x 35 x 50 cm). In the ORM test session, the retention of object information in memory was evaluated after a 24-h delay. The average total exploration time in mice was 22.28 ± 4.51 s, and similar to rats, a DI above 0.66 indicated that the animals were able to retain information on the objects in memory.

Y-maze test

We used the Y-maze method for hippocampally relevant spatial tasks, as previously described by Conrad et al. (26). Briefly, the rats were placed in the starting arm and allowed to explore 1 arm (familiar arm) of the Y maze for 15min while the assigned novel arm was inaccessible. The visual cues in the novel arm were not visible. After a 24-h delay, the rats were again placed into the starting arm and were allowed to freely explore both the familiar and novel arms. The percentage of time spent in the novel arm was analyzed. The average total exploration time of vehicle- and RGS14 lentivirus–treated animals was 220.15±4.94 and 211.48±4.61 s, respectively.

Morris water maze test

Spatial memory was tested according to the open-field water maze procedure, as previously described by Morris, with some modifications (27). Briefly, an escape platform was hidden 2 cm below the water surface in a fixed location in one of the 4 quadrants of the water maze. During 4 d of training, the animals were required to locate the submerged invisible platform by using distal extramaze cues. The animals were subjected to four 60 s trials each day. The time to reach the hidden platform(latency) was measured using a computerized tracking system (Ethovision XT; Noldus Information Technology, Leesburg, VA, USA). For another data set, 24h after d 1 or d4 of training, the animals were subjected to a probe session in which the platform was removed from the pool, and the rats were allowed to search for it for 30 s. An analysis of swimming time in the quadrant in which the platform had been located on the previous day was performed.

Taste discrimination test

Prior to experimentation, water-deprived rats were acclimated to drinking from 2 water bottles (A and B) for 10 min on 3 consecutive days. A record of daily liquid consumption was kept throughout the experiment. On the day of exposure to a taste, the rats were given saccharine solution (0.4 g/L) in both bottles for 10 min, and immediately after saccharine consumption, they were injected intraperitoneally with 0.1 M LiCl solution to evoke a weak conditioned taste aversion (CTA). After a delay of 60min or 24 h, the rats were exposed to saccharine solution (familiar taste) in 1 bottle (A) and 0.16Msodium chloride saline solution (novel taste) in another bottle (B) for 10 min. The amount of salt consumption (novel taste) in these animals was calculated. The average total exploration time of vehicle- and RGS14 lentivirus–treated animals was 165.41±6.47 and 162.88±5.41 s, respectively.

Odor memory test

Food-restricted rats were trained to dig in a small cup (3.5 cm diameter x 3 cm height) filled with scented sand and find a hidden half piece of Froot Loops cereal (Kellogg's, Battle Creek, MI, USA). An apparatus with 2 compartments of 50 x 50 x 60 cm each separated by a guillotine door was used for the behavioral test. During sample exposure, the rats were allowed to explore the starting compartment for 30 s, and the door was then opened. The animal crossed to the choice compartment (CC), and the door was closed. In the CC, there were 3 cups filled with scented sand with the same odor with a buried reward in all of them. However, the rats were allowed to eat the reward from only 1 cup. After a delay of 1 h or 24 h, a choice test was performed; in the CC, 1 cup had the familiar odor to which the rat had been exposed and contained a reward, and the other 2 cups had distinct novel odors and contained no reward. A correct response was recorded when the animal was able to find the reward in the correct cup without digging in the incorrect cups.

Tactile memory test

This test was conducted in a modified elevated plus maze in which the entire surface of each arm was covered with glass plates containing embossed patterns with different textures. The entire experiment took place in the dark under red light to prevent the rats from using the visual cues of the embossed patterns. On the day of the test, the rats explored both arms of the elevated plus maze containing identical textures for 5 min. After a delay of 30 min or 24 h, one of the arms was replaced with a new texture, and the rats were allowed to explore both the new and previously exposed (familiar) textures. The time of exploration in the new and familiar arms was recorded.

Temporal memory test

The CTA paradigm was used for this test. Water-deprived animals were given water daily in the morning (8:00 AM) and saccharine solution (0.4 g/L) at night (6:00 PM) for 10 min. On the third morning, the animals were given saccharine solution instead of water, and immediately after their drinking session, they received an intraperitoneal injection of lithium chloride (0.1 M) to induce a weak CTA. At night on the day of conditioning, the animals were given water. The day after conditioning, the animals were given water in the morning and at night. Afterward, during 5 consecutive days of extinction, the animals were allowed to drink water in the morning and saccharine solution at night. On the day of the test, in the morning, when conditioning occurred, the animals were exposed to saccharine solution. Daily saccharine solution intake was recorded throughout the experiment, and the percentage of saccharine intake was analyzed.

ORM test in AD mice

The ORM test in AD mice was very similar to that in BDNF mice with some exceptions (28). In the exploration session, the mice were exposed twice for 10 min each with 2 identical objects 10 min apart.

Statistics

The results were plotted and tested for statistical significance using Prism 7 (GraphPad Software, La Jolla, CA, USA). The samples were tested for normality using the Shapiro-Wilk normality test. Paired comparisons were analyzed using 2-tailed, paired Student's t tests (normally distributed) and Mann-Whitney tests (not normally distributed). Multiple comparisons with single variables were analyzed using 1-way ANOVA with post hoc Tukey's test. For multiple comparisons with more than 1 variable, a 2-way ANOVA with post hoc Sidak's test was used. All data in the figures are presented as means ± SEM.

RESULTS

$RGS14_{414}$ gene treatment in the PRh induces enhanced recognition memory

The PRh is a brain area that has been considered crucial for ORM processing in the brain (29, 30). Therefore, we tested whether $RGS14_{414}$ protein overexpression in the PRh can cause ORM enhancement. For this study, a lentivirus containing the $RGS14_{414}$ gene was

delivered into this area of the brain in Wistar Han rats for expression of the $RGS14_{414}$ protein, and after 3 wk, the memory status of these animals was evaluated by their performance on the ORM task, which is considered a hallmark test of recognition memory (31). When normal untreated 3-mo-old rats were exposed to an object for 3 min, they were able to retain information about the object in memory after a delay of 15, 30, or 45 min but not after a delay of 60 min or 24 h (Fig. 1A; 15, 30, and 45 min vs. 60 min or 24 h; 1-way ANOVA with Tukey's multiple comparisons test, $P<0.0001$). However, rats treated with the $RGS14_{414}$ gene in the PRh (RGS-PRh rats) were able to retain the same object information in memory after a delay of 60 min or 24 h (Fig. 1B; RGS14 vs. vehicle at 60 min or 24 h; 2-way ANOVA with Sidak's multiple comparisons test, $P< 0.0001$). Animals treated with lentivirus vehicle (vehicle), saline solution or even lentivirus containing RGS12, a protein that belongs to the same family as $RGS14_{414}$, did not induce the rats to retain this object information in memory (Fig. 1B; vehicle vs. saline or RGS12 at 60 min and 24 h; 2-way ANOVA with Sidak's multiple comparisons test, $P>0.05$), and the performance of these rats in the test was similar to that of untreated rats (60 min and 24 h in Fig. 1A). After behavioral studies, the brains of these animals were processed to evaluate the surface area affected by $RGS14_{414}$ gene injection. Both images of a coronal brain section after immunostaining with antibodies specific for RGS14 (Fig. 1C) and a depiction of coronal brain sections after analysis of serial sections, which both showed maximum expansion of the $RGS14_{414}$ protein in the PRh (drawings in red color in Fig. 1D), indicated that $RGS14_{414}$ protein expression was confined to the PRh area. We further observed that elimination of PRh neurons in RGS-PRh rats by the administration of Ox7-SAP, a saporin-based immunotoxin that causes selective eradication of neurons and does not affect other brain structures or passing nerve fibers (32, 33), completely abolished the memory-enhancing effect of $RGS14_{414}$ gene treatment (Fig. 1E; no Ox7 vs. Ox7 in the PRh; 1-way ANOVA with Tukey's multiple comparisons test, $P <0.0001$). In contrast, Ox7-SAP administration in the parietal cortex of RGS-PRh rats showed no effect on memory enhancement (Fig. 1E; Ox7 in the parietal cortex vs. Ox7 in the PRh; 1-way ANOVA with Tukey's multiple comparisons test, P.0.05). In the same line, a treatment of $RGS14_{414}$ gene into parietal cortex, another brain area, produced no effect on ORM (Fig. 1E; RGS14 in parietal cortex). Together, these results suggest that $RGS14_{414}$-mediated ORM enhancement was specific to PRh. Furthermore, a cresyl violet-stained brain section showing the affected area after lesions were produced by Ox7-SAP immunotoxin injection in the PRh (arrow in Fig. 1F) demonstrated a substantial loss of neurons in this area. These findings from lesion studies suggest that the memory-enhancing activity observed in RGS-PRh rats is mediated by PRh neurons at the site of $RGS14_{414}$ gene injection.

$RGS14_{414}$ gene treatment promotes neuronal arborization

We observed that the effect of$RGS14_{414}$ treatment on ORM was not short-lived. Instead, the effect endured for a long time (Supplemental Fig. S1). We posited that this long-lasting effect was due to permanent structural changes caused by neuronal remodeling. Hence, the brains of $RGS14_{414}$-treated rats were subjected to Golgi-Cox silver staining, and an analysis of cell body neurite outgrowth in both pyramidal and nonpyramidal

neurons and proliferation in dendritic branches and synapses of pyramidal neurons was performed. Both pyramidal and nonpyramidal neurons from $RGS14_{414}$-treated rat brains showed robust neuronal arborization (Fig. 2Ai, ii). $RGS14_{414}$ treatment produced an increase in neurites originating from the cell body of pyramidal neurons (Fig. 2B; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.007), and this effect was more pronounced for neurites of nonpyramidal neurons (Fig. 2B; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.0004). In contrast to neurites, $RGS14_{414}$ treatment caused a robust increase in dendritic branching of pyramidal neurons, with almost twice the amount of dendritic branching in vehicle-treated animals (Fig. 2B; 2-way ANOVA with Sidak's multiple comparisons test, P<0.0001). However, the analysis of neurite branching showed no difference in pyramidal neurons or nonpyramidal neurons. Altogether, these results suggest that RGS14414 treatment induced overwhelming increases in dendritic branching of pyramidal neurons and in cell body neurites of nonpyramidal neurons. Furthermore, we observed a substantial shift in the proportion of pyramidal neurons with a higher amount of branching (Supplemental Fig. S2). In vehicle-treated control animals, the number of dendritic branches per neuron was up to 8; however, this figure increased to 15 in RGS14-treated rats. In contrast, an analysis of dendritic synaptic density of pyramidal neurons per micrometer of length showed no difference in the total number of synapses or in their subtypes, which included thin, mushroom, and stubby (Fig. 2C; vehicle vs. RGS14 in total, thin, mushroom, or stubby synapses; 2-way ANOVA with Sidak's multiple comparisons test, P>0.05). These findings indicate that after $RGS14_{414}$ treatment, the spatial distribution of synapses in dendrites was unchanged. Instead, there was an almost 2-fold increase in the surface area of dendrites. Therefore, $RGS14_{414}$-treated animals had twice as many synapses as vehicle-treated animals.

BDNF mediates neuronal arborization and ORM enhancement

Permanent structural plasticity that causes a long-term change in memory functions, such as that observed in $RGS14_{414}$-treated rats (Fig. 1B), has often been associated with neurotrophic factors (34, 35). Thus, we next studied the effect of $RGS14_{414}$ treatment on FGF2, NGF, and BDNF, which are neurotrophic factors that are abundant in the brain and related to structural plasticity and memory. We first evaluated the mRNA levels of these neurotrophic factors, and we then evaluated their protein levels by Western blotting. qRT-PCR analysis revealed that the mRNA levels of BDNF in RGS-treated animals were 2.4- fold higher than those in vehicle-treated control animals (Fig. 3A; vehicle vs.RGS14; 2-tailed, paired Student's t test, P = 0.0002). However, in contrast to BDNF, there was no effect on the mRNA levels of the other 2 neurotrophic factors, NGF and FGF-2 (Fig. 3A). Therefore, these results indicate that $RGS14_{414}$ treatment exerts a strong but selective effect on BDNF mRNA levels. We next examined the effect of this treatment on pro-BDNF (BDNF) protein levels. For this purpose, we extracted brains at 7, 14, and 21d after $RGS14_{414}$ gene treatment and processed them for Western blot analysis. The plot of the optical density values of the protein bands from the Western blot in the top panel of Fig. 3B shows increases in pro-BDNF levels after 7 (19.96 ± 2.72% in bottom panel of Fig. 3B; vehicle vs. RGS14; 2-way ANOVA with Sidak's multiple comparisons test, P=0.0002) and 14 (40.32±3.35% in bottom panel of Fig. 3B; 2-way ANOVA with Sidak's

multiple comparisons test, $P < 0.0001$) days of $RGS14_{414}$ treatment. However, the effect was more prominent after 21 d of treatment, when pro-BDNF protein expression was 71.25 ± 7.77% higher than that of the vehicle-treated control (bottom panel of Fig. 3B; 2-way ANOVA with Sidak's multiple comparisons test, $P < 0.0001$). This marked increase in pro-BDNF protein expression at 14 and 21 d after treatment coincides well with the period during which the emergence of ORM enhancement has been observed in RGS-PRh animals. The effect of $RGS14_{414}$ treatment on memory enhancement in animals generally appears 18 d after treatment (Supplemental Fig. S3).

Afterward, to evaluate the extent of BDNF involvement in RGS14-mediated neuronal arborization and ORM enhancement, heterozygous mutant mice of BDNF ($BDNF^{-}$) were included in this study. Similar to rats, wild-type mice subjected to RGS14414 gene treatment in the PRh showed increases in both dendritic branching in pyramidal neurons (Fig. 3C; wild type + vehicle vs. wild type + RGS14; 2- way ANOVA with Sidak' smultiple comparisons test, $P < 0.0001$) and neurites in nonpyramidal neurons (Fig. 3C; wild type + vehicle vs. wild type + RGS14; 2-wayANOVA with Sidak's multiple comparisons test, $P = 0.0008$). However, when the same treatment was performed in $BDNF^{-}$ mice, the effect on neuronal proliferation disappeared, and the $BDNF^{-}$ mice showed dendritic branching in pyramidal neurons and neurite numbers in nonpyramidal neurons similar to those of wild-type vehicle-treated mice (Fig. 3C; wild type + vehicle vs. $BDNF^{-}$ + RGS14; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.8228$ for pyramidal dendritic branching; $P = 0.8747$ for nonpyramidal neurites). In addition to neuronal proliferation, we further studied the effect of $RGS14_{414}$ gene treatment on ORM enhancement in $BDNF^{-}$ mice. Treatment of wild-type mice with the $RGS14_{414}$ gene showed ORM enhancement similar to that in rats (Fig. 3D; wild type + vehicle vs. wild type + RGS14; 1-way ANOVA with Tukey's multiple comparisons test, $P < 0.001$); however, the same treatment in BDNF– mice abrogated the memory-enhancing effect (Fig. 3D; wild type + vehicle vs. $BDNF^{-}$ + RGS14; 1-way ANOVA with Tukey'smultiple comparisons test, P.0.05). In contrast to mice subjected to $RGS14_{414}$ gene treatment, wild-type mice that were untreated or treated with vehicle exhibited no effect on ORM. The complete loss of RGS-mediated neuronal arborization and ORM enhancement after the deletion of BDNF in mice suggests that $RGS14_{414}$ exerts its function primarily through the regulation of BDNF signaling.

$RGS14_{414}$ gene treatment augments spatial, taste, odor, and tactile memory

Considering that the ORM-enhancing effect of $RGS14_{414}$ is facilitated through the activation of BDNF, a protein that is implicated in the processing of memory for all senses (36, 37), we posited that in addition to boosting object memory, treatment with the $RGS14_{414}$ gene might also boost spatial, taste, odor, and tactile memory in a similar manner. In this study, we targeted brain areas for $RGS14_{414}$ gene treatment that have previously been demonstrated to be involved in the processing of each of these types of memory. Accordingly, we selected the CA1 area for spatial memory (38), the PRh for taste and tactile memory (39), and the prelimbic cortex for odor memory (40). The effect of $RGS14_{414}$ on spatial memory was first investigated by gene treatment in the CA1 area. For this study, we applied 2 different approaches: the Y-maze (Fig. 4Ai) and the Morris

water maze (MWM) (Fig. 4Aii, iii). In the Y-maze test, the rats were first exposed to an arm for 15 min and then allowed to freely explore that same familiar arm and a novel arm after a delay of 1 or 24 h. Animals that explored significantly longer in the novel arm were considered to have been able to retain the spatial information for the familiar arm in memory, and animals that explored both arms equally were unable to retain such information. Based on this view, we found that normal untreated rats could retain the information related to the familiar arm in memory after a delay of 1 h, and they were unable to retain this information after a delay of 24 h (Fig. 4Ai; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.0005$ for untreated 1 h; $P = 0.9997$ for untreated 24 h). However, rats treated with $RGS14_{414}$ in the CA1 area could remember the familiar arm even after a delay of 24 h (RGS14 at 24 h in Fig. 4Ai; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.0014$). Treatment with vehicle instead of the $RGS14_{414}$ gene did not produce this memory-enhancing effect (vehicle at 24 h in Fig. 4Ai). In the MWM test, the time to reach the hidden platform (latency) was analyzed. A shorter time to reach the platform indicated that the animals were able to retain information about the platform location in memory, which helped them locate the platform and perform better in this test. We observed that RGS14-treated animals took notably less time to reach the hidden platform on d 2 and 3 of training than did the vehicle-treated rats (Fig. 4Aii; 2-wayANOVA with Sidak's multiple comparisons test, $P = 0.0058$ for d 2; $P = 0.0011$ for d 3). This enhanced performance was not attributed to a difference in swimming speed because both the RGS14 and vehicle groups displayed similar speeds when swimming (Supplemental Fig. S4). Thus, $RGS14_{414}$- treated animals learned faster as a result of their higher memory capacity. This finding was further confirmed by a test of how well the animals remembered the location of the hidden platform 24 h after d 1 of training. We found that RGS14 animals spent more time in the quadrant area in which the hidden platform was located than vehicle treated animals (24 h after d 1 of training in Fig. 4Aiii; 2- way ANOVA with Sidak's multiple comparisons test, $P = 0.0087$). This difference was not observed after d 4 of training. To study the memory-enhancing effect of $RGS14_{414}$ on taste memory, $RGS14_{414}$ gene treatment was performed in the PRh, and the rats were exposed to a saccharine solution after water deprivation. Immediately after drinking the saccharine solution, the rats were injected intraperitoneally with 0.1 M LiCl solution to evoke a weak CTA. After a delay of 60 min or 24 h, the animals were exposed to the CTA-related saccharine solution (familiar taste) and a new taste solution to test whether these animals could retain the conditioning information for the familiar taste in memory. We considered animals that drank more of the new taste solution to have remembered being conditioned to the familiar taste, and animals that drank both solutions equally did not remember being conditioned. Based on this criterion, we found that after CTA, normal untreated rats could retain the conditioning information for the familiar solution in memory for 60 min but not for 24 h (Fig. 4B; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.0001$ for untreated 60min; $P = 0.7420$ for untreated 24 h).However, when the animals were treated with the $RGS14_{414}$ gene, they were able to retain the same information for 24 h (Fig. 4B; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.004$). In contrast, animals treated with vehicle showed no effect

on taste memory. Therefore, our results suggest that $RGS14_{414}$ treatment enhances taste memory.

Similarly, to test the effect of $RGS14_{414}$ treatment on odor memory, $RGS14_{414}$ injection was performed in the prelimbic cortex. The rats were first trained to dig in scented sand cups to find a hidden piece of Froot Loops cereal and to discriminate the rewarded odor from unrewarded odors. During the test, the animals were exposed to an odor with a reward, and after a delay of 1 or 24 h, they were presented with 3 odors. Digging in the cup with the reinforced odor was considered to be the correct response, and in our experiments, more than 80% of the correct responses are considered to occur because the animals remember the reinforced odor information. Following this benchmark, we observed that normal untreated animals were able to retain the information related to the reinforced odor after a delay of 1 h, but they were unable to retain this information after a delay of 24 h (Fig. 4C; 1 h untreated vs. 24 h untreated; 1-way ANOVA with Tukey's multiple comparisons test, $P < 0.0001$). However, in contrast to normal untreated animals, animals treated with the $RGS14_{414}$ gene exhibited enhanced odor memory, and RGS14-treated rats were capable of remembering the reinforced odor even after a delay of 24 h (Fig. 4C; 24 h vehicle vs. 24 h RGS14; 1-way ANOVA with Tukey's multiple comparisons test, $P < 0.0001$). Furthermore, treatment with vehicle showed no effect on odor memory, and the performance of such rats was similar to that of untreated rats (24 h).

The effect of $RGS14_{414}$ on tactile memory was determined in rats injected with the $RGS14_{414}$ gene in the PRh area of the brain using a texture discrimination task. In this test, we employed a customized elevated plus maze in which only 2 arms were open, and the entire surface of each arm was covered with replaceable textured glass plates. The animals were left for 5 min to freely explore both arms, which had identical textures. After a delay of 30 min or 24 h, the animals were exposed to arms in which 1 arm was replaced with a new texture, and the texture of the other arm remained the same as in the previous session (familiar). Animals that explored significantly longer in the arms with the new texture than in the arm with the familiar texture were considered to have retained the information related to the familiar texture in memory. We observed that normal untreated rats could retain the texture information in memory for 30 min; however, they failed to retain this information for 24 h (Fig. 4D; 2-way ANOVA with Sidak's multiple comparisons test, $P = 0.0015$ for 30 min untreated; $P = 0.5641$ for 24 h untreated). In contrast to normal untreated animals, animals treated with RGS14414 were able to remember this information after a 24-h delay (Fig. 4D; 2-way ANOVA with Sidak's multiple comparisons test, $P<0.0001$). Furthermore, treatment with vehicle produced no change in this type of memory (Fig. 4D; 2-way ANOVA with Sidak's multiple comparisons test, $P =0.9056$), and the vehicle-treated animals exhibited behavior similar to that of normal untreated animals.

Recovery of recognition, spatial, and temporal memory in aging rats

Remarkably, $RGS14_{414}$ gene treatment was able to enhance various forms of memory. In patients or individuals who display symptoms of memory deficit due to multiple etiologies, recognition, spatial, and temporal memory are principally affected (41).

Therefore, we next examined whetherRGS14_{414} gene treatment in the PRh could reverse memory deficits observed during normal aging and in AD, the 2most representative conditions in which deficits in memory have consistently been reported (3, 42). For this study, we used normal aging Wistar rats and AD mice. The studies were first performed in aging rats. We found that untreated young rats at 3mo of age (young untreated) were able to retain information about an object in memory; however, when these rats reached 18 mo of age (aged untreated), a noticeable decrease in ORM was observed, with recognition memory falling to a level at which the rats were unable to recall the same information (Fig. 5A; young untreated vs. aged untreated; 1-way ANOVA with Tukey's multiple comparisons test, $P < 0.0001$). After treating these memory-deficient aged rats with the RGS14_{414} gene, full recovery of ORM was observed (Fig. 5A; aged+RGS14vs. aged untreated; 1-wayANOVAwith Tukey's multiple comparisons test, $P < 0.0001$), and the performance of the rats reached a level similar to that of young untreated animals. In contrast, vehicle treatment did not produce any effect on recognition memory, and the ORM levels of vehicle-treated rats were similar to those of untreated aged rats (Fig. 5A; aged + vehicle vs. aged untreated; 1-way ANOVA with Tukey's multiple comparisons test, $P >0.05$).

To evaluate the effect of treatment on spatial memory, we used a paradigm of behavioral performance in the MWM test. After 4 d of training, untreated aged rats required more time than untreated young rats to find the hidden platform in the MWM (d 4 in Fig. 5B and the same data expanded in Fig. 5C; young untreated vs. aged untreated; 1-way ANOVA with Tukey's multiple comparisons test, $P = 0.0043$). However, when aged rats were treated with the RGS14_{414} gene in the PRh, their times to reach the hidden platform were reduced noticeably (d 4 in Fig. 5B and the same data expanded in Fig. 5C; aged untreated vs. aged + RGS14; 1-way ANOVA with Tukey's multiple comparisons test, $P = 0.0014$), and their performance was similar to that of untreated young rats. In contrast, treatment with vehicle in aged rats exerted no effect on performance (d 4 in Fig. 5B and the same data expanded in Fig. 5C; aged vehicle vs. aged untreated; 1-way ANOVA with Tukey's multiple comparisons test, $P > 0.05$). Thus, our results indicate that RGS14_{414} treatment led to the recovery of spatial memory in aging rats and that aged RGS14-treated animals were able to retain information in the brain related to cues that guided the rats to locate the platform more efficiently than their untreated and vehicle-treated counterparts. Furthermore, unexpectedly, the effect of RGS14414 treatment was so potent that in addition to the recovery of spatial memory (Fig. 5B; aged + vehicle vs. aged + RGS14; 2-wayANOVA with Sidak's multiple comparisons test, $P = 0.0014$ for d 2; $P < 0.0001$ for d 3), the performance of aged RGS14- treated animals in the MWM test was far superior to that of normal untreated young rats from d 2 of training. This superior performance of aged RGS-treated animals was not due to a difference in swimming speed (Supplemental Fig. S5).

To test the effect of RGS14_{414} treatment on the recovery of temporal memory in aging rats, the CTA paradigm was applied for behavioral assessment associated with the time of conditioning. Water-deprived rats were exposed to saccharine solution for 10 min at night for 2 consecutive days. On d 3, saccharine solution was given in the morning, and immediately after drinking the solution, the rats were injected intraperitoneally with

0.1MLiCl solution to evoke conditioning to saccharine intake at this time of day. From d 2 to d 6 after conditioning, these animals were given saccharine solution at night. On d 7 (test day), the animals were exposed to saccharine solution in the morning to test whether they were able to retain information related to the conditioning performed in the morning after saccharine intake. We considered that those animals that drank a significantly reduced volume of saccharine remembered the morning conditioning and that rats that drank more than they did during the previous session did not retain this information. Under this condition, we compared the saccharine intake of young untreated rats to that of the previous day and found that aged, untreated rats consumed considerably higher volumes of saccharine solution than young rats (Fig. 5D; 2- way ANOVA with Sidak's multiple comparisons test, P = 0.0059). These results suggest that young untreated animals could retain the information associated with morning saccharine intake conditioning and that aged rats were unable to retain this information. However, when these memory-deficient aged rats were treated with the RGS14414 gene in the PRh, they were able to retain the same information in their memory; accordingly, reduced saccharine consumption by these aged RGS-treated animals was observed (Fig. 5E; aged + RGS14; extinction d 6 vs. test day; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.0089). However, this RGS14-mediated recuperation of temporal memory in aging rats was not observed when the rats were treated with vehicle (Fig. 5E, aged + vehicle; extinction d 6 vs. test day; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.8786).

Recovery of recognition, spatial, and temporal memory in AD mice

As described for aging rats, the ORM test was used to evaluate recognition memory, the MWM test was used to evaluate spatial memory, and the CTA paradigm was used to evaluate temporal memory in AD mice. In the ORM test, AD mice showed a noticeable ORM deficit at the age of 4 mo (Fig. 6A; AD vs. wild type; 1-way ANOVA with Tukey's multiple comparisons test, P < 0.0001). Treatment of these memory-deficient AD mice with the RGS14414 gene in the PRh led to full recovery of recognition memory (Fig. 6A; 1-way ANOVA with Tukey's multiple comparisons test, P<0.0001). RGS14-treatedAD mice showed ORM levels similar to those of wild-type mice. However, vehicle treatment showed no effect on recognition memory, and the ORM levels of vehicle treated mice were similar to those of untreated AD mice (Fig. 6A; AD + vehicle vs. AD; 1-way ANOVA with Tukey's multiple comparisons test, P > 0.05). After behavioral studies, the brains of these mice were processed to evaluate the surface area affected by $RGS14_{414}$ gene injection. A depiction of coronal brain sections showing maximum expansion of the $RGS14_{414}$ protein in the PRh (Supplemental Fig. S6, drawings in red) indicated that $RGS14_{414}$ protein expression was limited to the PRh area.

In the MWM test, we found that AD mice required more time to reach the platform than did wild-type mice on d 2 (Fig. 6B; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.0220), d 3 (P = 0.0023), and d 4 (P = 0.0207) of training. However, when these AD mice were treated with the RGS14414 gene in the PRh, their performance improved remarkably on d 2 (Fig. 6B; AD + vehicle vs. AD + RGS14; 2-way ANOVA with Sidak's multiple comparisons test, P = 0.0023), d 3 (P = 0.0002) and d 4 (P = 0.0011).

The overall performance of RGS14-treated AD mice was better than that of wild-type mice. AD mice treated with vehicle did not show any improvement in performance (Fig. 6B; AD + vehicle vs.AD after 1, 2, 3, or 4 d; 2-way ANOVA with Sidak's multiple comparisons test, $P > 0.05$). These results indicate that $RGS14_{414}$ treatment in AD mice led to the recovery of spatial memory to a level similar to that observed in aged rats.

Morning conditioning to saccharine intake was evaluated in a manner similar to that described for the aged rat experiments. Aversion conditioning in all groups of mice generated a marked but consistent reduction in saccharine consumption (after conditioning in Fig. 6C). After 6 d of extinction, on the day of the test, the wild-type mice were exposed to saccharine solution in the morning, and when aversion conditioning was invoked, the mice consumed less solution (Fig. 6C; extinctiond6 vs. test; 2-wayANOVA with Tukey's multiple comparisons test, $P = 0.0062$); however, AD mice showed no aversive response to saccharine solution exposure (Fig. 6C; extinction d 6 vs. test; 2- way ANOVA with Tukey's multiple comparisons test, $P = 0.7518$). These data suggest that AD mice were unable to retain information associated with morning conditioning to saccharine solution intake in their memory. Treatment of these memory-deficient AD mice with $RGS14_{414}$ caused the recovery of temporal memory, and these mice consumed markedly less saccharine solution (Fig. 6C; extinction d 6 vs. test; 2-way ANOVA with Tukey's multiple comparisons test, $P = 0.0004$). This recovery of temporal memory was also evident from the results of the test day, on which saccharine intake in RGS14-treated AD mice was considerably lower than that of vehicle-treated AD mice or untreated AD mice (Fig. 6C; test; AD+ RGS14 vs. AD + vehicle or AD; 2-way ANOVA with Tukey's multiple comparisons test, $P < 0.0001$). Nevertheless, this recuperation of temporal memory was not observed when AD mice were treated with vehicle (Fig. 6C; AD + vehicle; extinction d 6 vs. test; 2-way ANOVA with Tukey's multiple comparisons test, $P = 0.6879$).

DISCUSSION

Our findings demonstrate that treatment with the RGS14414 gene not only enhanced various types of memory but also rescued deficits in recognition, spatial, and temporal memory, which are kinds of episodic memory that are primarily affected in patients or individuals showing memory deficiency (41). This memory enhancing effect of RGS14414 was associated with BDNF mediated up-regulation of arborization in pyramidal and nonpyramidal neurons. We found that in pyramidal neurons, branching was more dominant in dendrites, which are structures that innervate to other brain areas (43). However, in nonpyramidal neurons, branching was prevalent in cell body neurites, which are structures that are involved in local circuits. In general, this branching generated an ;2-fold increase in the synapse number of both types of neurons. Considering that pyramidal neurons are primarily involved in excitatory functions and nonpyramidal neurons are primarily involved in inhibitory cortical functions, this surge in the number of synapses could have caused an increase in neuronal connections within excitatory and inhibitory components of neuronal circuits. These observations indicate that $RGS14_{414}$ induces well-balanced neuronal circuit reorganization. An imbalance between excitatory

and inhibitory circuit activities has been linked to neurologic and psychiatric diseases. Therefore, a balanced increase in neuronal circuitry could promotemore efficient memory related information processing and further facilitate memory formation in the brain. Similarly, it has been shown that long-term memory formation is critically associated with synaptic remodeling, including growth in number of synapses (44, 45). Memory is thought to be processed through interconnected brain circuits that are formed by the participation of distinct brain areas, and memory deficits occur as a consequence of reduced activity within these circuits (46–48). Consistent with this observation, poorer performance in patients with memory deficiency has been shown to be associated with decreased functional activity in neural networks (46, 49). Therefore, it is likely that synaptic reorganization caused by neuronal arborization could restore memory by reviving activity in these interconnected networks. An increase in the activity of these circuits could thus explain the enhanced memory in normal animals and the rescued memory in memory-deficient animal models. The results obtained after injection in different brain areas indicate that $RGS14_{414}$ was adequate for the activation of single (1 type of memory) and multiple (several types of memory) memory circuits. We found that the PRh is a brain area in which object, spatial, taste, tactile, and temporal memory converge, and treatment of this area with $RGS14_{414}$ produced enhancements in all of these types of memory. Therefore, our studies of gains in memory function suggest that the PRh might function as one of the main memory hubs, where information is exchanged among various kinds of memory, and that this area can be targeted selectively to rescue episodic memory deficits. This finding represents an important milestone in our understanding of how the function of a specific memory circuit or multiple memory circuits can be restored.

We found that increases in neuronal arborization and memory enhancement were dependent on BDNF signaling because deletion of BDNF in mice ablated both of these activities. Similar to our observation, BDNF has been shown to play crucial roles in neuronal growth, branching, and the formation of synaptic connections and is essential for long-term memory (50). A surge in BDNF was observed within a week of $RGS14_{414}$ treatment; however, ORM enhancement was first detected after 18 d of treatment. Therefore, we believe that the increase in BDNF initially induced neuronal arborization and the generation of new synapses, which then enforced the reorganization of neuronal connectivity. This neuronal circuit reorganization could have served as a structural substrate for the memory-enhancing effect of $RGS14_{414}$. This circuit reorganization seems to occur within 4 wk of the injection because further neuronal arborization was undetectable beyond this period. There was no difference in neuronal arborization between 4 and 12 wk after the injection. In addition, a considerable drop in BDNF levels at 12 wk after the injection coincided well with the inactivity in neuronal arborization during this period. Thus, the memory-enhancing effect appears to emerge when novel connections are established, and neural circuits are reorganized.

Previous *in vivo* studies have reported that Ras binding domain R1 within RGS14 interacts with Ras and mediates the integration of Ras/Raf/MAPK signaling events (51). Evidence in the literature suggests that Ras/Raf/MAPK signaling converges on the ERK1/2-CREB pathway, and activation of the ERK1/2-CREB pathway causes an

increase in BDNF transcription (50). Although our $RGS14_{414}$ gene lacks the RGS domain, it contains the Ras binding domain R1. Therefore, it is likely that overexpression of $RGS14_{414}$ promotes enhanced MAPK signaling, which could in turn facilitate ERK1/2-CREB-mediated BDNF transcription and augment BDNF levels, as observed in animals after RGS14414 treatment. An upsurge in BDNF within 1wk after RGS14414 treatment further supports this notion. Considering that BDNF and its receptor tropomyosin receptor kinase B (TrkB) are crucial for neuronal plasticity (52) and dendrite development (53), and inactivity in autocrine BDNF-TrkB signaling reduces spine and dendrite growth, we propose that the initial increase in BDNF levels through the ERK1/2-CREB pathway could amplify its effect through autocrine activity in the BDNF-TrkB pathway. This autocrine pathway is linked to several important signaling pathways, including phospholipase C gamma–inositol trisphosphate–diacylglycerol (PLCg-IP3-DAG), MEK1/2-ERK1/2, and protein kinase B–mammalian target of rapamycin (AKT-mTOR) Therefore, the autocrine action of BDNF can amplify the initial effect through the activation of multiple signaling pathways. This possibility could explain the robust effect of $RGS14_{414}$ treatment on multiple types of memory and the reversal of episodic memory deficits. It is well documented in the literature that both mRNA and protein levels of BDNF are significantly reduced during normal aging and in AD (54–58). Therefore, the recovery of memory deficits after $RGS14_{414}$ gene treatment in rodent models of aging and AD was likely due to the restoration of BDNF function.

Substances known to enhance memory include hormones, neurotransmitters, neuropeptides, and metabolic substrates, and these factors produce very distinct effects on various types of memory (10). In contrast to $RGS14_{414}$, which, in addition to enhancing multiple kinds of memory, has shown strong efficacy in reversing memory deficits in memory-deficient models, no other memory enhancers have demonstrated the same level of effectiveness in memory enhancement; more importantly, these factors have not been reported to have any effect on the recovery of memory deficits. Our work suggests that RGS14414 is unique and is not only adequate for the recovery of types of memory that are particularly affected in patients with memory deficits but also useful as a general memory enhancer for the enhancement of multiple kinds of memory.

ACKNOWLEDGMENTS

The authors thank Zafar I. Bashir [Medical Research Council (MRC) Centre for Synaptic Plasticity, University of Bristol, Bristol, United Kingdom] for reading and providing critical feedback during the preparation of the manuscript. This research was supported by grants from the Ministerio de Economía y Competitividad (BFU2013-43458-R) and Junta de Andalucia (P12-CTS-1694 and PI-0542-2013 to Z.U.K.). The authors thank C. Vivar Rios [Laboratory of Neurobiology, Centro de Investigaciones Medico Sanitaria (CIMES), University of Malaga] for help preparing graphs and figures for this manuscript. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Z. U. Khan developed the overall research concept and the project; M. Masmudi-Martín, I. Navarro-Lobato, M. F. López-Aranda, J. C. López, and Z. U. Khan designed the experiments; M. Masmudi-Martín, I. Navarro-Lobato, M. F. López-Aranda, G. Delgado, E. Martín-Montañez, M. E. Quiros-Ortega, M. Carretero-Rey, L. Narváez, M. F. Garcia-Garrido, S. Posadas, I. Jiménez-Recuerda, and P. Granados-Durán performed the experiments; J. F. López- Téllez, E. Blanco, and J. Paez-Rueda assisted with the experiments; and M. Masmudi-Martín, I. Navarro-Lobato, M. F. López-Aranda, and Z. U. Khan wrote the manuscript.

REFERENCES

1. Bishop, N. A., Lu, T., and Yankner, B. A. (2010) Neural mechanisms of ageing and cognitive decline. *Nature* **464**, 529–535
2. Khan, Z. U., Martín-Montañez, E., Navarro-Lobato, I., and Muly, E. C. (2014) Memory deficits in aging and neurological diseases. *Prog. Mol. Biol. Transl. Sci.* **122**, 1–29
3. Walsh, D. M., and Selkoe, D. J. (2004) Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron* **44**, 181–193
4. Bibb, J. A., Mayford, M. R., Tsien, J. Z., and Alberini, C. M. (2010) Cognition enhancement strategies. *J. Neurosci.* **30**, 14987–14992
5. Baxter, M. G., Lanthorn, T. H., Frick, K. M., Golski, S., Wan, R. Q., and Olton, D. S. (1994) D-cycloserine, a novel cognitive enhancer, improves spatial memory in aged rats. *Neurobiol. Aging* **15**, 207–213
6. Kalisch, R., Holt, B., Petrovic, P., De Martino, B., Klöppel, S., Büchel, C., and Dolan, R. J. (2009) The NMDA agonist D-cycloserine facilitates fear memory consolidation in humans. *Cereb. Cortex* **19**, 187–196
7. Lynch, G. (2006) Glutamate-based therapeutic approaches: ampakines. *Curr. Opin. Pharmacol.* **6**, 82–88
8. Linssen, A. M., Vuurman, E. F., Sambeth, A, and Redel, W. J. (2012) Methylphenidate produces selective enhancement of declarative memory consolidation in healthy volunteers. *Psychopharmacology (Berl.)* **221**, 611–619
9. Pepeu, G., and Giovannini, M. G. (2010) Cholinesterase inhibitors and memory. *Chem. Biol. Interact.* **187**, 403–408
10. Stern, S. A., and Alberini, C. M. (2013) Mechanisms of memory enhancement. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **5**, 37–53
11. López-Aranda, M. F., Acevedo, M. J., Carballo, F. J., Gutiérrez, A., and Khan, Z. U. (2006) Localization of the GoLoco motif carrier regulator of G-protein signalling 12 and 14 proteins in monkey and rat brain. *Eur. J. Neurosci.* **23**, 2971–2982
12. Lee, S. E., Simons, S. B., Heldt, S. A., Zhao, M., Schroeder, J. P., Vellano, C. P., Cowan, D. P., Ramineni, S., Yates, C. K., Feng, Y., Smith, Y., Sweatt, J. D., Weinshenker, D., Ressler, K. J., Dudek, S. M., and Hepler, J. R. (2010) RGS14 is a natural suppressor of both synaptic plasticity in CA2 neurons and hippocampal-based learning and memory. *Proc. Natl. Acad. Sci. USA* **107**, 16994–16998

13. Evans, P. R., Parra-Bueno, P., Smirnov, M. S., Lustberg, D. J., Dudek, S. M., Hepler, J. R., and Yasuda, R. (2018) RGS14 restricts plasticity in hippocampal CA2 by limiting postsynaptic calcium signaling. *eNeuro* **5**, ENEURO.0353–17.2018
14. Kimple, R. J., De Vries, L., Tronchère, H., Behe, C. I., Morris, R. A., Gist Farquhar, M., and Siderovski, D. P. (2001) RGS12 and RGS14 GoLoco motifs are G alpha(i) interaction sites with guanine nucleotide dissociation inhibitor Activity. *J. Biol. Chem.* **276**, 29275–29281
15. Siderovski, D. P., Diversé-Pierluissi, M., and De Vries, L. (1999) The GoLoco motif: a Galphai/o binding motif and potential guanine-nucleotide exchange factor. *Trends Biochem. Sci.* **24**, 340–341
16. Mucke, L., Masliah, E., Yu, G. Q., Mallory, M., Rockenstein, E. M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000) High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058
17. Palop, J. J., Jones, B., Kekonius, L., Chin, J., Yu, G. Q., Raber, J., Masliah, E., and Mucke, L. (2003) Neuronal depletion of calcium-dependent proteins in the dentate gyrus is tightly linked to Alzheimer's disease-related cognitive deficits. *Proc. Natl. Acad. Sci. USA* **100**, 9572–9577
18. Chin, J., Palop, J. J., Puoliväli, J., Massaro, C., Bien-Ly, N., Gerstein, H., Scearce-Levie, K., Masliah, E., and Mucke, L. (2005) Fyn kinase induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease. *J. Neurosci.* **25**, 9694–9703
19. Paxinos, G., and Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*, 4th ed., Academic Press, Cambridge, MA, USA
20. Paxinos, G., and Franklin, K. B. J. (2001) *The Mouse Brain in Stereotaxic Coordinates*, 2nd ed., Academic Press, Cambridge, MA, USA
21. López-Aranda, M. F., López-Téllez, J. F., Navarro-Lobato, I., Masmudi-Martín, M., Gutiérrez, A., and Khan, Z. U. (2009) Role of layer 6 of V2 visual cortex in object-recognition memory. *Science* **325**, 87–89
22. Ennaceur, A., and Delacour, J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav. Brain Res.* **31**, 47–59
23. Khan, Z. U., Mrzljak, L., Gutierrez, A., de la Calle, A., and Goldman-Rakic, P. S. (1998) Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc. Natl. Acad. Sci. USA* **95**, 7731–7736
24. Morrison, J. H., and Baxter, M. G. (2012) The ageing cortical synapse: hallmarks and implications for cognitive decline. *Nat. Rev. Neurosci.* **13**, 240–250
25. Khan, Z. U., Fernando, L. P., Escribá, P., Busquets, X., Mallet, J., Miralles, C. P., Filla, M., and De Blas, A. L. (1993) Antibodies to the human gamma 2 subunit of the gamma-aminobutyric acidA/benzodiazepine receptor. *J. Neurochem.* **60**, 961–971
26. Conrad, C. D., Galea, L. A., Kuroda, Y., and McEwen, B. S. (1996) Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. *Behav. Neurosci.* **110**, 1321–1334

27. Morris, R. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* **11**, 47–60
28. Escribano, L., Simón, A. M., Pérez-Mediavilla, A., Salazar-Colocho, P., Del Río, J., and Frechilla, D. (2009) Rosiglitazone reverses memory decline and hippocampal glucocorticoid receptor down-regulation in an Alzheimer's disease mouse model. *Biochem. Biophys. Res. Commun.* **379**, 406–410
29. Brown, M. W., and Aggleton, J. P. (2001) Recognition memory: what are the roles of the perirhinal cortex and hippocampus? *Nat. Rev. Neurosci.* **2**, 51–61
30. Murray, E. A., and Richmond, B. J. (2001) Role of perirhinal cortex in object perception, memory, and associations. *Curr. Opin. Neurobiol.* **11**, 188–193
31. Winters, B. D., Saksida, L. M., and Bussey, T. J. (2008) Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neurosci. Biobehav. Rev.* **32**, 1055–1070
32. Traissard, N., Herbeaux, K., Cosquer, B., Jeltsch, H., Ferry, B., Galani, R., Pernon, A., Majchrzak, M., and Cassel, J. C. (2007) Combined damage to entorhinal cortex and cholinergic basal forebrain neurons, two early neurodegenerative features accompanying Alzheimer's disease: effects on locomotor activity and memory functions in rats. *Neuropsychopharmacology* **32**, 851–871
33. Nolan, B. C., and Freeman, J. H., Jr. (2005) Purkinje cell loss by OX7-saporin impairs excitatory and inhibitory eyeblink conditioning. *Behav. Neurosci.* **119**, 190–201
34. Waterhouse, E. G., and Xu, B. (2009) New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. *Mol. Cell. Neurosci.* **42**, 81–89
35. McAllister, A. K., Katz, L. C., and Lo, D. C. (1999) Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* **22**, 295–318
36. Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J. I., Goldin, A., Izquierdo, I., and Medina, J. H. (2008) BDNF is essential to promote persistence of long-term memory storage. *Proc. Natl. Acad. Sci. USA* **105**, 2711–2716
37. Andero, R., Choi, D. C., and Ressler, K. J. (2014) BDNF-TrkB receptor regulation of distributed adult neural plasticity, memory formation, and psychiatric disorders. *Prog. Mol. Biol. Transl. Sci.* **122**, 169–192
38. Griffin, A. L., Eichenbaum, H., and Hasselmo, M. E. (2007) Spatial representations of hippocampal CA1 neurons are modulated by behavioral context in a hippocampus-dependent memory task. *J. Neurosci.* **27**, 2416–2423
39. Cacciamani, L., and Likova, L. T. (2016) Tactile object familiarity in the blind brain reveals the supramodal perceptual-mnemonic nature of the perirhinal cortex. *Front. Hum. Neurosci.* **10**, 92
40. Tronel, S., and Sara, S. J. (2003) Blockade of NMDA receptors in prelimbic cortex induces an enduring amnesia for odor-reward associative learning. *J. Neurosci.* **23**, 5472–5476
41. Brickman, A. M., and Stern, Y. (2009) Aging and memory in humans. In *Encyclopedia of Neuroscience* (L. R. Squire, ed.), pp. 175–180, Elsevier, Amsterdam
42. Robitsek, R. J., Fortin, N. J., Koh, M. T., Gallagher, M., and Eichenbaum, H. (2008) Cognitive aging: a common decline of episodic recollection and spatial memory in rats. *J. Neurosci.* **28**, 8945–8954

43. Spruston, N. (2008) Pyramidal neurons: dendritic structure and synaptic integration. *Nat. Rev. Neurosci.* **9**, 206–221
44. Lamprecht, R., and Le Doux, J. (2004) Structural plasticity and memory. *Nat. Rev. Neurosci.* **5**, 45–54
45. Bailey, C. H., Kandel, E. R., and Harris, K. M. (2015) Structural components of synaptic plasticity and memory consolidation. *Cold Spring Harb. Perspect. Biol.* **7**, a021758
46. Dickerson, B. C., and Eichenbaum, H. (2010) The episodic memory system: neurocircuitry and disorders. *Neuropsychopharmacology* **35**, 86–104
47. Samson, R. D., and Barnes, C. A. (2013) Impact of aging brain circuits on cognition. *Eur. J. Neurosci.* **37**, 1903–1915
48. Hof, P. R., and Morrison, J. H. (2004) The aging brain: morphomolecular senescence of cortical circuits. *Trends Neurosci.* **27**, 607–613
49. Daselaar, S. M., Veltman, D. J., Rombouts, S. A., Raaijmakers, J. G., and Jonker, C. (2003) Deep processing activates the medial temporal lobe in young but not in old adults. *Neurobiol. Aging* **24**, 1005–1011
50. Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A., and Moryś, J. (2018) BDNF: a key factor with multipotent impact on brain signaling and synaptic plasticity. *Cell. Mol. Neurobiol.* **38**, 579–593
51. Shu, F. J., Ramineni, S., and Hepler, J. R. (2010) RGS14 is a multifunctional scaffold that integrates G protein and Ras/Raf MAPkinase signalling pathways. *Cell. Signal.* **22**, 366–376
52. Harward, S. C., Hedrick, N. G., Hall, C. E., Parra-Bueno, P., Milner, T. A., Pan, E., Laviv, T., Hempstead, B. L., Yasuda, R., and McNamara, J. O. (2016) Autocrine BDNF-TrkB signalling within a single dendritic spine. *Nature* **538**, 99–103
53. Wang, L., Chang, X., She, L., Xu, D., Huang, W., and Poo, M. M. (2015) Autocrine action of BDNF on dendrite development of adult-born hippocampal neurons. *J. Neurosci.* **35**, 8384–8393
54. Connor, B., Young, D., Yan, Q., Faull, R. L., Synek, B., and Dragunow, M. (1997) Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Brain Res. Mol. Brain Res.* **49**, 71–81
55. Hock, C. H., Heese, K., Olivieri, G., Hulette, C. H., Rosenberg, C., Nitsch, R. M., and Otten, U. (2000) Alterations in neurotrophins and neurotrophin receptors in Alzheimer's disease. *J. Neural Transm. Suppl.* **59**, 171–174
56. Michalski, B., and Fahnestock, M. (2003) Pro-brain-derived neurotrophic factor is decreased in parietal cortex in Alzheimer's disease. *Brain Res. Mol. Brain Res.* **111**, 148–154
57. Narisawa-Saito, M., Wakabayashi, K., Tsuji, S., Takahashi, H., and Nawa, H. (1996) Regional specificity of alterations in NGF, BDNF and NT-3 levels in Alzheimer's disease. *Neuroreport* **7**, 2925–2928
58. Peng, S., Wuu, J., Mufson, E. J., and Fahnestock, M. (2005) Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *J. Neurochem.* **93**, 1412–1421

FIGURES

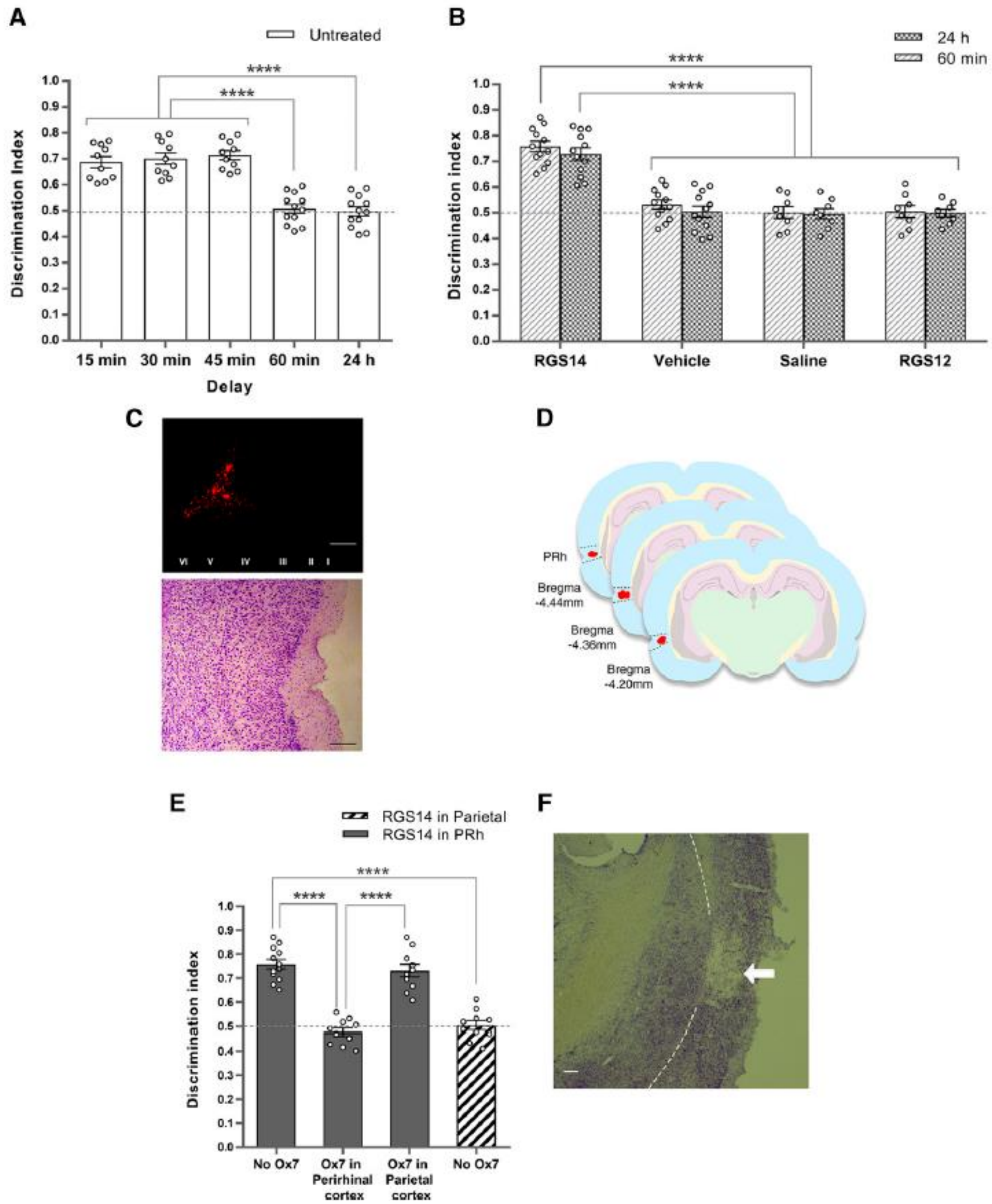


Figure 1. RGS14414 gene treatment induces enhanced ORM. A) Three-month-old normal untreated rats were able to retain object information in memory for 15, 30, and 45 min; however, they were unable to retain such information after 60 min and 24 h. B) RGS14414 gene treatment in the PRh (RGS-PRh) induced ORM enhancement that could be observed at 60 min and 24 h, and treatment with vehicle, saline, and RGS12, another gene from the same family, caused no effect. C) The top image shows the immunolabeled RGS14414 protein in red, and the bottom image shows the cresyl violet-stained brain section showing the PRh area. Cortical layers (I-VI) are indicated in the top image, for which the scale bar is 100 mm. D) Drawing showing the localization of the RGS14414

protein (red) obtained after the analysis of serial coronal sections from 5 RGS-PRh animals and representing the maximum expansion area in all 3 sections. E) Elimination of PRh neurons in RGS-PRh animals by the selective immunotoxin Ox7-SAP completely abolished ORM enhancement (Ox7 in PRh), whereas Ox7 treatment in the parietal cortex of the same RGS-PRh animals showed no effect (Ox7 in parietal cortex). RGS14414 gene treatment in the parietal cortex also showed no effect on ORM (striped the PRh (arrow). The dotted white line separates the cortical area from the subcortical area. Scale bar, 320 mm. Dotted lines across A and B indicate the threshold at which (0.5 DI and below) the animals were unable to retain object information in memory. ****P < 0.0001 [1-way ANOVA with post hoc Tukey's test (A, E), 2-way ANOVA with post hoc Sidak's test (B)].

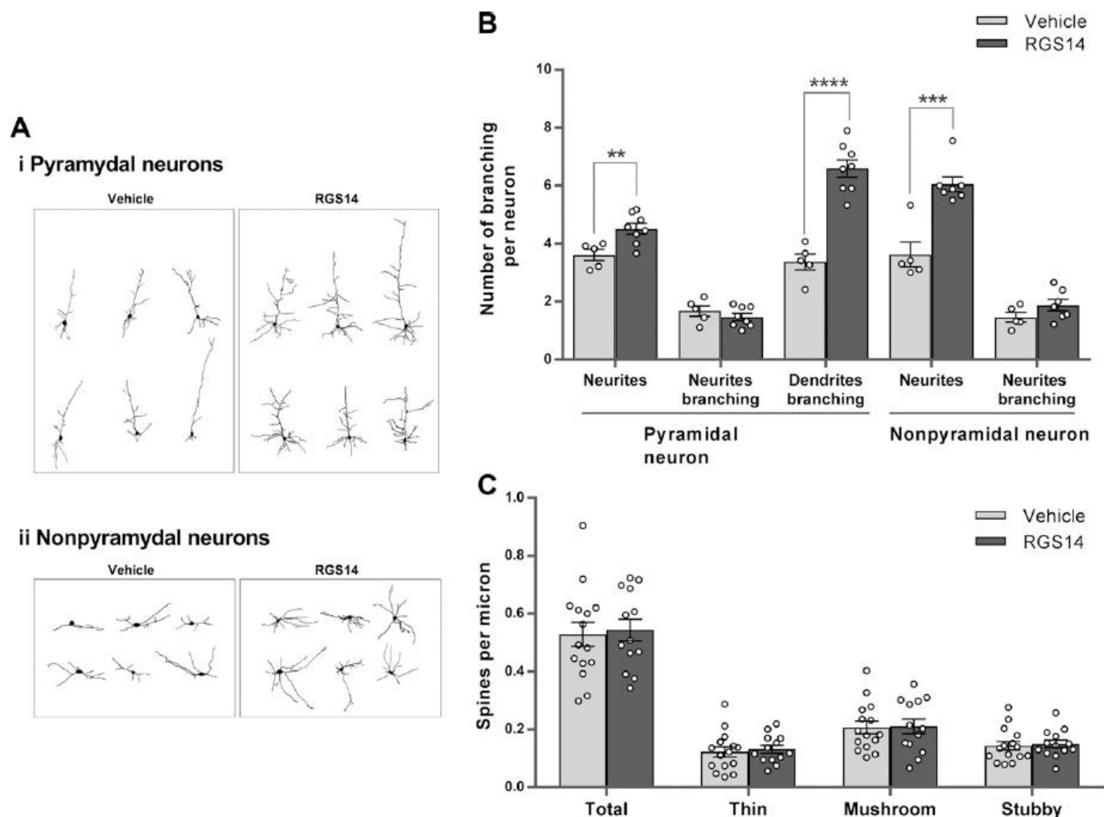


Figure 2. RGS14414 treatment induces robust neuronal arborization. A) Neuronal drawings representing examples of pyramidal neurons (i) and nonpyramidal neurons (ii) from RGS14- and vehicle-treated rats. B) Neuronal branching analyzed in 79 pyramidal and 55 nonpyramidal neurons. RGS-treated animals showed robust increases in the number of neurites and the amount of dendritic branching in pyramidal neurons. However, the effect was more prominent for dendritic branching. In contrast, the effect was more dominant for neurites in nonpyramidal neurons. C) Dendritic spine density of pyramidal neurons analyzed from a 1052-mm length containing ;515 spines in each condition showed no difference in total spines or in subtypes of spines, indicating that

synapse occupancy per micrometer distance remains the same. However, due to a 2-fold increase in dendritic surface area, the total number of synapses in RGS14414 animals was 2 times greater than that in vehicle-treated animals. **P ≤ 0.007 (2-way ANOVA with post hoc Sidak's test); ***P ≤ 0.0004 (2-way ANOVA with post hoc Sidak's test); ****P < 0.0001 (2-way ANOVA with post hoc Sidak's test).

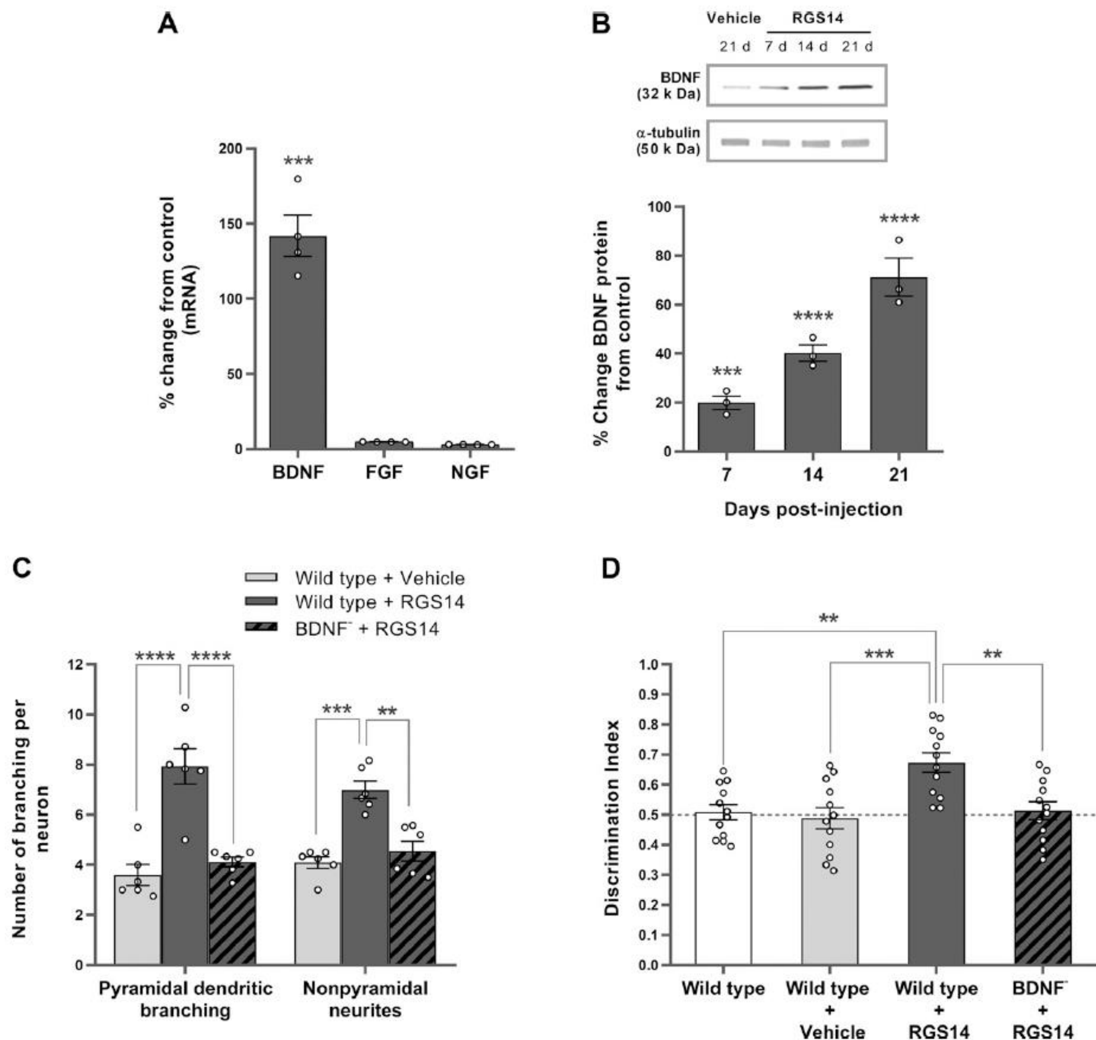
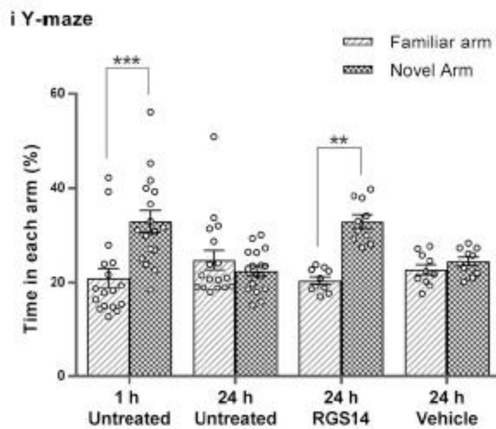


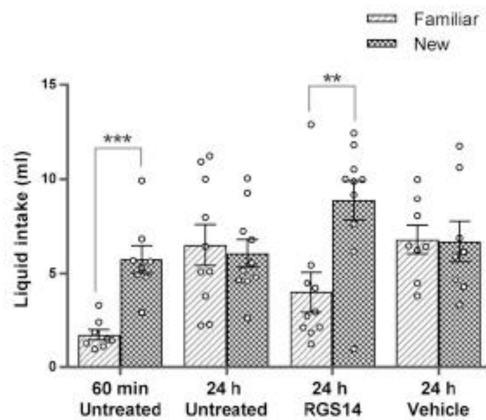
Figure 3. RGS14$_{414}$ mediates its effect through BDNF. A) qRT-PCR analysis shows that RGS14 gene treatment increased BDNF mRNA levels but not FGF or NGF mRNA levels. B) The top panel represents an example of a Western blot showing BDNF protein expression after 7, 14, and 21 d of RGS14 gene treatment, and the bottom panel represents an analysis of normalized optical density values of Western blot bands. BDNF protein expression reached 71.25% after 21 d of treatment. C) RGS14$_{414}$ treatment in wild-type mice induced robust increases in pyramidal dendritic branching and neurites in nonpyramidal neurons; however, this effect disappeared when the same treatment was performed in BDNF-deficient (BDNF2) mice. D) Wild-type mice displayed enhanced ORM after treatment with RGS14 (wild type + RGS14), whereas this memory-enhancing

effect of RGS14 was absent in BDNF2 mice (BDNF2 + RGS14). The dotted line across the figure indicates the threshold at which (≤0.5 DI) the animals were unable to retain object information in memory. **P < 0.01 [2-way ANOVA with post hoc Sidak's test (C), 1-way ANOVA with post hoc Tukey's test (D)]; ***P ≤ 0.001 [2-tailed, paired Student's t test (A), 2-way ANOVA with post hoc Sidak's test (B, C), 1-way ANOVA with post hoc Tukey's test (D)]; ****P < 0.0001 [2-tailed, paired Student's t test (A), 2-way ANOVA with post hoc Sidak's test (B, C)].

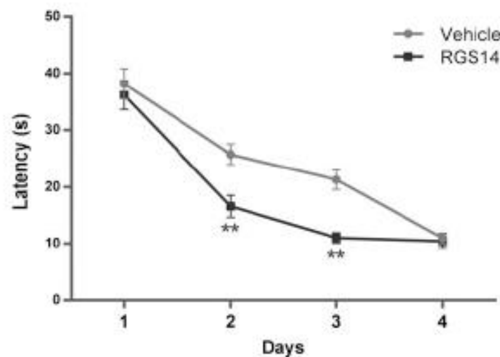
A Spatial memory



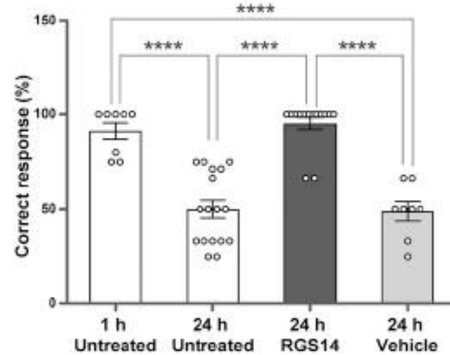
B Taste memory



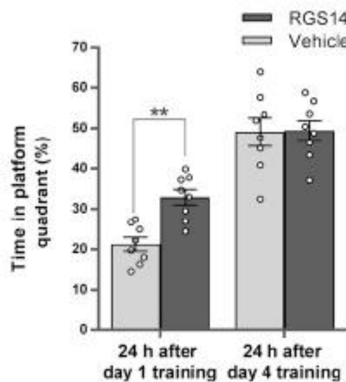
ii Morris water maze. Latency



C Odor memory



iii Morris water maze. Information retention



D Tactile memory

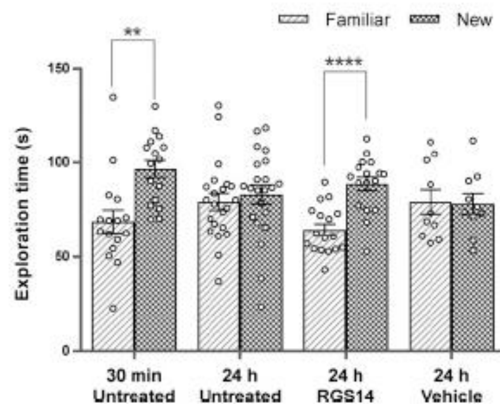


Figure 4. $RGS14_{414}$ treatment enhances various types of memory. A) Spatial memory was tested with 2 approaches: the Y-maze and the MWM. i) Results of the Y maze, in which normal untreated rats were able to retain information about a previously explored familiar arm in memory for 1 h but not for 24 h. However, when these rats were treated with RGS14, they could retain the same information in memory for 24 h. ii, iii) Results of the MWM test. Rats treated with RGS14 performed much better than vehicle-treated animals, and their time to reach the platform was significantly reduced after d 2 of training. A test of information retention related to the platform location 24 h after the first day of training showed that the RGS animals spent more time in the quadrant in which the platform was localized; however, after 4 d of training, when both groups learned the location of platform, their performance was equivalent to that of vehicle-treated animals. B) Taste memory results. Normal untreated rats were able to retain information concerning a taste (familiar) associated with mild conditioning that produced pain and internal malaise in memory for 60 min, and their intake of a new taste was significantly higher, but the rats failed to exhibit these behaviors after 24 h. However, animals treated with RGS14 were able to retain the same taste information in memory for 24 h. C) Odor memory results. Normal untreated rats were able to retain information on an odor for 1 h but not for 24 h. However, similar to taste memory, rats treated with RGS14 could recall the same odor after 24 h. D) Tactile memory results. Normal untreated animals were able to retain information on a texture (familiar) in memory for 30 min and therefore explored a novel texture for a longer time, but they were unable to retain this information after 24 h. However, after receiving RGS14 treatment, these animals could retain the same information on texture in memory for 24 h. **$P < 0.01$ [2-way ANOVA with post hoc Sidak's test (A, B, D)]; ***$P \leq 0.001$ [(2-way ANOVA with post hoc Sidak's test (A, B, D)] ****$P < 0.0001$ [1-way ANOVA with post hoc Tukey's test (C), 2-way ANOVA with post hoc Sidak's test (D)].

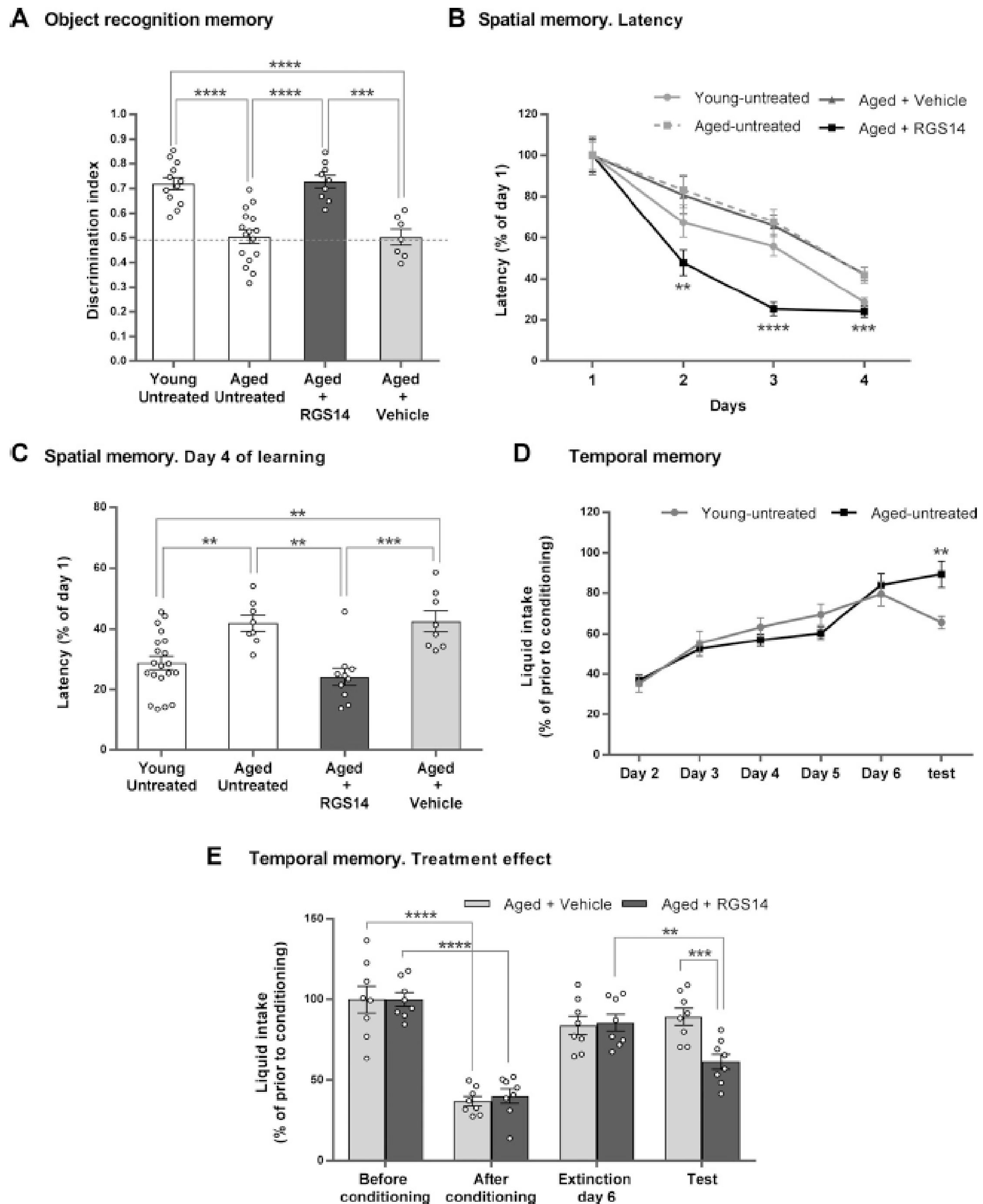
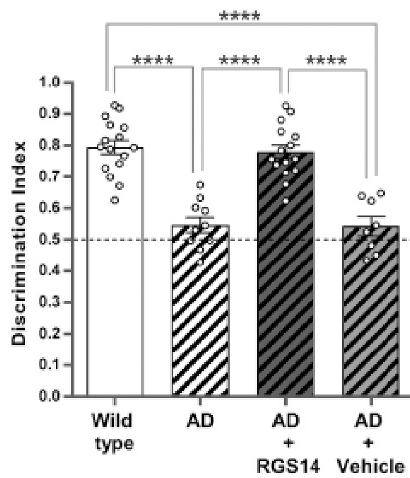
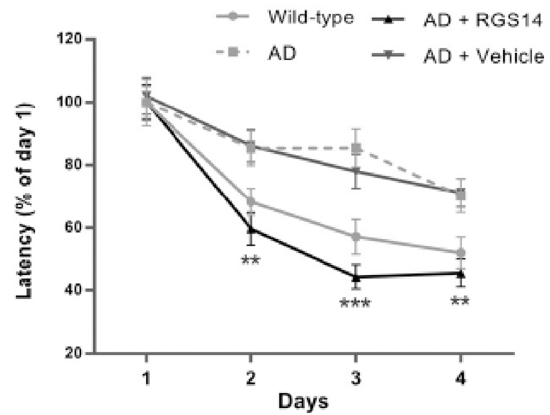


Figure 5. Recovery of recognition, spatial, and temporal memory in aging rats. A) In the recognition memory test, when young untreated rats were exposed to an object, they could retain the object information in memory; however, aged untreated rats were unable to retain this information. $RGS14_{414}$ gene treatment in the PRh of these memory-deficient aged rats led to full recovery of this recognition memory. In contrast, vehicle treatment did not produce any effect. The dotted line across the figure indicates the threshold at which (0.5 DI and below) the animals were unable to retain object information in memory. B, C) Results of the spatial memory test. Even after 4 d of learning in the MWM (C), aged untreated rats needed much more time to reach the platform than young untreated rats, and their performance was significantly poorer. However, when these aged

rats were treated with the $RGS14_{414}$ gene, they showed far superior performance after d 2 of training than young untreated animals (B). D, E) Results of the temporal memory test. In contrast to aged untreated rats that failed to remember the morning aversion conditioning to saccharine liquid, young untreated rats were able to retain this information in memory even after 6 d of extinction (D, day of test). Treatment with RGS14414 in these memory-deficient aged rats caused the recovery of temporal memory (E, extinction d 6 vs. day of test), and their response to saccharine conditioning in the morning was the same as that in young untreated animals. After aversion conditioning, a similar reduction in saccharin liquid intake was observed in both groups (E). **P < 0.01 [1-way ANOVA with post hoc Tukey's test (C), 2-way ANOVA with post hoc Sidak's test (B, D, E)]; ***P < 0.001 [1-way ANOVA with post hoc Tukey's test (A, C), 2-way ANOVA with post hoc Sidak's test (B, E)]; ****P < 0.0001 [1-way ANOVA with post hoc Tukey's test (A), 2-way ANOVA with post hoc Sidak's test (E)].





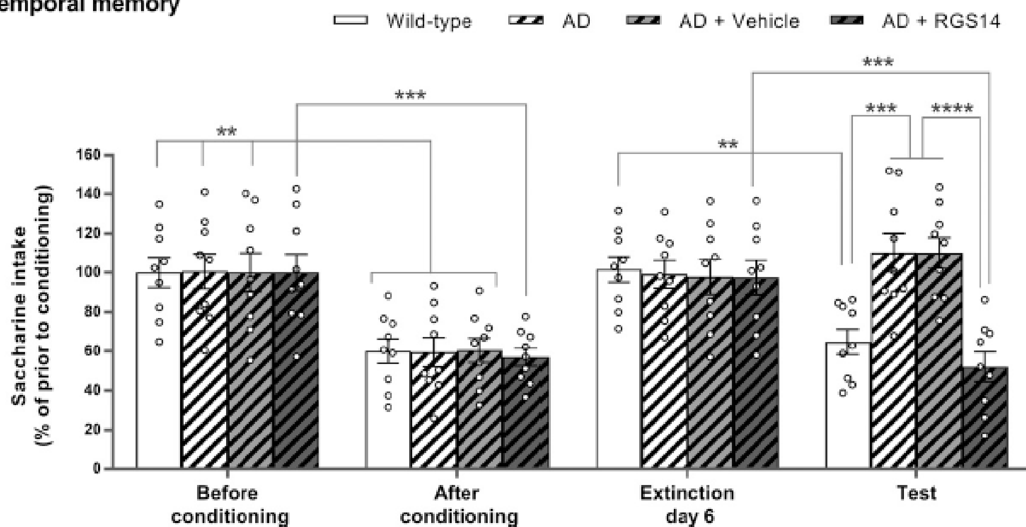


Figure 6. Recovery of recognition, spatial, and temporal memory in AD mice. A) AD mice showed substantial ORM loss compared to that of wild-type mice. Akin to aging rats, $RGS14_{414}$ gene treatment in the PRh of AD mice led to a full recovery of recognition memory. The dotted line across the figure indicates the threshold (#0.5 DI) at which the animals were unable to retain object information in memory. B) Spatial memory results in AD mice. Similar to aged rats, AD mice needed more time to reach the platform than wild-type mice, and their performance was noticeably worse from d 2 of training. However, treatment with $RGS14_{414}$ in AD mice caused recovery of spatial memory. These AD + RGS14 mice showed better performance in the MWM than wild-type mice on d 2 of training. C) Temporal memory results in AD mice. Similar to aged rats, AD mice were unable to retain the information on morning aversive conditioning to saccharine even after 6 d of extinction (AD bars of extinction d 6 vs. test), whereas wild-type mice could retain this information in memory (wild-type bars of extinction d 6 vs. test). However, when these memory-deficient AD mice were treated with $RGS14_{414}$, they showed a recovery of temporal memory, and these mice consumed much less saccharine

on the day of the test (AD + RGS14 bars of extinction d 6 vs. test). **$P < 0.01$ [2-way ANOVA with post hoc Sidak's test (B), 2-way ANOVA with post hoc Tukey's test (C)]; ***$P < 0.001$ [2-way ANOVA with post hoc Sidak's test (B), 2-way ANOVA with post hoc Tukey's test (C)]; ****$P < 0.0001$ [1-way ANOVA with post hoc Tukey's test (A), 2-way ANOVA with post hoc Tukey's test (C)].