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Chemogenetic stimulation of hippocampal adult-born neurons improves long-term memory accuracy

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Hippocampal adult neurogenesis is involved in many memory processes from learning, to remembering and forgetting. However, whether or not the stimulation of adult neurogenesis can improve memory performance remains unclear. Here, using a chemogenetic approach that combines selective tagging and specific activation of distinct adult-born neuron populations, we demonstrate that this activation can improve remote memory accuracy and strength. These results open up new avenues for remedying memory problems that may arise over time.

In the adult hippocampus, new neurons are continuously generated throughout life. Because the hippocampus is critical for memory, many studies have been devoted to understanding the role of adult neurogenesis in memory processes^{1,2}. Manipulating adult neurogenesis impacts hippocampal-dependent learning, in particular spatial navigation³ and contextual fear conditioning⁴ and many memory processes, such as memory retrieval⁵, behavioral pattern separation⁶, and system consolidation⁷,

reconsolidation⁸ and forgetting⁹. These studies suggest that memory deficits observed in memory pathologies could be linked to a disturbance of adult neurogenesis.

Nevertheless, relatively few studies have shown any beneficial effect on memory resulting from the stimulation of adult neurogenesis other than those resulting from the genetic enhancement of the neurogenic pool^{9,10}. Because it is known that adult neurogenesis is homeostatically regulated³, the addition of new neurons can lead to compensatory mechanisms¹¹. Thus, knowledge about the effect of specifically activating a given population of new neurons, already present at the time of learning, on subsequent memory processes is lacking. Here we tested whether chemogenetic stimulation of neuronal activity of new neurons during retrieval could enhance long-term memory retention in two different hippocampal-dependent tasks.

Results

We first wanted to analyze long-term spatial memory in the water maze (WM). Towards this end, 2 months-old adult rats were trained to locate a hidden platform in a fixed location in the water maze (WM) for 6 days. Then two groups of animals were tested respectively 48 hours or 4 weeks later (Fig 1a). The results showed that, at both tests, latency to cross the position of where the platform was during training was not different between groups and not different from the latency on the last training day (Fig 1b). This shows that the spatial information was retained at both delays. However, when we considered the number of annulus crossings or the percentage of time spent in the target zone, results showed that the performances were impaired when rats were tested 4 weeks after training. Both annulus crossings and time spent in zone were higher in the target zone compared to those in the other zones at 48h, whereas no difference was

observed when memory was tested at 4 weeks (Fig 1 c,d). This demonstrates that the passage of time disrupted spatial memory accuracy.

To further determine whether stimulation of adult-born neurons could improve memory accuracy, we developed a GFP retrovirus (RV) into which we inserted a DREADD-Gs construct (Gs-GFP- RV)(see Suppl Methods). This tool acts initially by infecting new granular cells at their birth. Several weeks later, when they are fully integrated into the network, these same cells can then be activated upon binding of the synthetic ligand Clozapine-N-Oxide (CNO). To ensure that Gs-GFP-RV injections into the dentate gyrus (DG) had no impact on new neuron survival, rats were injected in the DG with either the Gs-GFP-RV or with a control retrovirus (GFP-RV). At the same time, both groups of animals were injected with bromodeoxyuridine (BrdU) to study cell genesis. Six weeks later, the animals were sacrificed in order to quantify the number of surviving BrdU-positive cells. No differences were observed between the groups, demonstrating that injection of the Gs-GFP-RV has no impact on BrdU-labeled cell survival (Fig. 2a-b). To further confirm that CNO injections specifically activate Gs-GFP-RV cells, rats were injected with Gs-GFP-RV in the left DG and with a control GFP-RV in the right DG. Six weeks later, all animals received an IP injection of CNO (1mg/Kg) and were sacrificed two hours later (Fig. 2c). We determined the activation of the infected cells by analyzing the expression of the immediate early gene Zif268 in the GFP labeled cells. We found that 98.8% of the cells infected with the Gs-GFP-RV were activated by CNO compared to 1.72% of the cells injected with a Ctrl-retrovirus (Fig. 2d). Finally, we performed whole cell recordings of Gs-GFP-RV or control GFP-RV-infected cells (Fig. 2e) and assessed changes in cell excitability after CNO application (Fig. 2f). Local perfusion of CNO quickly and reversibly enhanced Gs-GFP-RV-infected cell activity, which was seen by an increase

in both resting potential and action potential firing rate (Fig. 2g). No such effect was seen in GFP-RV-infected cell activity (Fig. 2h).

We then sought to determine whether stimulating adult-born neurons during retrieval could promote memory retention. We have recently shown that adult-born neurons that were immature (1 week-old) or mature (6 weeks-old) at the time of training were both activated by remote memory retrieval. However, only the immature population was necessary for remote memory reconsolidation⁸. We therefore first focused on the population of neurons born one week before training (Fig 3a). We have previously shown that the survival and dendritic development of this latter population is increased by learning¹² and it has been hypothesized that these immature neurons could be primed by experience^{14,15}. Recent data showed that this immature population is required for maintaining the memory trace after retrieval⁸. However, whether the stimulation of neurons that were immature at the time of learning is capable of improving memory retrieval is not known. Two month-old rats were injected with either the Gs-GFP-RV or the GFP-RV. One week later, these rats were trained in the water maze (Fig 3a). All rats learned to find the platform (Fig 3b). Four weeks after training, we performed a remote retention test, one hour prior to which all rats received an IP injection of CNO. We found that stimulation of this population enhanced memory retention. Compared to GFP rats, the number of annulus crossings and the time spent in the target zone were higher in the Gs-GFP rats (Fig 3c,d,e). Gs-GFP, but not GFP, rats showed also a clear preference for the target zone compared to the others zones with performances significantly above the chance level. These results demonstrate that stimulation of adult-born neurons that were immature at the time of learning could enhance memory accuracy. To confirm that dentate granular neurons were correctly transduced, the number of GFP positive cells was estimated in the dentate gyrus. This

analysis showed that 3156 ± 295 cells were GFP positive and distributed along the DG septo-temporal axis (Fig3f).

We then performed the same experiment, but this time, we targeted the population of adult-born neurons that were mature (6 weeks-old) at the time of training (Fig 4a).

This specific population is known to be activated by spatial learning and retrieval and its pharmacological ablation disrupted spatial memory learning^{13,14}. We have also recently shown that chemogenetic silencing of this population impairs recent spatial memory retrieval⁸. Hence, two month-old rats were injected with either the Gs-GFP-RV or the GFP-RV. Six weeks later, they were trained in the water maze. All groups learned to find the platform (Fig 4b). Four weeks after training, we performed a remote retention test, one hour prior to which all rats received an IP injection of CNO (Fig 4a). Stimulation of mature adult-born neurons infected with the Gs-GFP-RV enhanced retention, since the Gs-GFP-RV, but not the GFP-RV, rats made more crossings and spent more time in the target zone compared to those in the other zones (Fig 4c,d,e). The neurons transduced by the Gs-GFP-RV (1303 ± 150 cells) were distributed along the DG septo-temporal axis (Fig 4f).

One could argue that the stimulation of the dentate gyrus, no matter the population targeted, could enhance memory retention. To determine whether the effect observed on memory was specific to the stimulation of adult-born neurons, we targeted dentate granular neurons generated postnatally. To do so, rats were injected with either the Gs-GFP-RV or the GFP-RV, 3 days after birth (P3). Then rats were trained in the WM at 2 months of age. Four weeks later, CNO was injected one hour before a probe test (Fig 5a). In this case, the stimulation of postnatally-generated granular neurons had no effect on memory retention (Fig 5b-e). We estimated the number of neurons transduced by the

Gs-GFP-RV and we found that 7588 ± 1255 cells were GFP positive and distributed along the DG septo-temporal axis (Fig 5f). These results indicate that the beneficial memory effects of chemogenetic stimulation does not depend on the number of DG cells transduced.

Altogether these data reveal that chemogenetic stimulation of adult-born neurons generated before learning, and not developmentally-born granular neurons, enhances remote memory accuracy which can be lost with the passage of time.

We then investigated whether chemogenetic stimulation of adult-born neurons could enhance long-term memory in another hippocampal-dependent task, i.e, contextual fear conditioning (CFC). Towards this end, two month-old rats were injected with either the Gs-GFP-RV or the GFP-RV. One week later, these rats were trained in contextual fear conditioning (Fig 6a). All rats received two mild foot-shocks over a period of 4 min (Fig 6b). During conditioning, both groups had the same level of freezing (Fig 6c). Five weeks later, when the targeted cells reached the age of 6 weeks-old, we performed a remote retention test, one hour prior to which all rats received an IP injection of CNO. Freezing was measured over 3 min (Fig 6b). The results showed the level of freezing was higher in the Gs-GFP rats compared to that of GFP control animals (Fig 6d). Finally, we estimated the number of neurons transduced by the Gs-GFP-RV and we found that 1799 ± 311 cells were GFP positive and distributed along the DG septo-temporal axis (Fig 6e,f). These data demonstrate that stimulating adult-born neurons that were present at the time of learning increases the strength of remote memory.

Discussion

Here, we show that stimulating adult-born neurons generated before learning can rescue memory accuracy in a navigational task and promote fear response. Our data underline a new role for a population of immature neurons at the time of learning. While this specific population is not activated by learning and is not necessary for recent memory retrieval⁸, its silencing results in disruption of memory maintenance after reactivation. Altogether, these results add to previous findings demonstrating that decreasing adult neurogenesis before training impairs remote but not recent spatial memory¹⁵ and suggest that adult-born neurons are important for consolidation, retrieval and reconsolidation.

Although activation of neurons which are immature at the time of learning leads to the reengagement of the network and eventually to memory retrieval, they do not meet the definition of “engram cells”¹⁶, because they are not activated by learning⁸. They are nonetheless undergoing structural changes in response to learning¹². As a result these immature cells become part of the engram network but further experiments targeting the mature cells that did encode the information are needed to understand how immature neurons could, together with the engram cells, be engaged in the engram network.

The stimulation of the population of neurons that were mature at the time learning also induces an increase of spatial memory accuracy. These neurons are activated by spatial learning and memory retrieval¹³ and their silencing disrupts recent memory recall⁸. We can speculate that this population was part of the initial engram that encoded the spatial information. However, remote memory stabilization after reactivation does not depend on this population⁸.

The positive effect observed on remote memory seems to be specific to adult-born neurons stimulation. In fact, stimulation of postnatally-generated cells does not improve memory accuracy. It should be noted that more neurons were transduced with the retroviruses in P3 pups than in adult rats, ruling out the hypothesis that the lack of effect observed could be due to a low number of neurons transduced. The results are consistent with previous finding showing that postnatally-generated neurons are not critical for spatial learning¹⁷, nor for recent¹⁸ or remote retrieval⁸. Together, these data confirm that postnatally-generated neurons are not involved in spatial learning in adult rats.

Finally, using another hippocampal-dependent task, i.e contextual fear conditioning, we found that stimulating the population of new neurons that was immature at the time of learning could enhance contextual fear response. This demonstrates that the beneficial effect of adult-born neurons stimulation is not restricted to spatial memory but may be generalized to hippocampal-dependent memory.

Altogether these findings promote the idea that adult neurogenesis is essential for memory accuracy¹⁹ and could be a valuable tool to compensate for the loss of precision and detail induced by the passage of time. Since the dentate gyrus is known to govern the reactivation of remote memory trace²⁰ and to diminish generalization of remote fear memories²¹, our results confirm that adult-born dentate neurons are key players in the role of the dentate network.

Finally, promoting memory is a relevant outcome when it comes to aging. During aging, not only the rate but also the responsiveness of adult hippocampal neurogenesis is altered^{22,23}. Here, we propose that increasing the activity of adult-born neurons is a promising strategy for prevention or treatment of memory loss.

Methods

Animals

A total of 134 male Sprague–Dawley rats (OFA, Charles Rivers, France) were used for these experiments. Rats weighing between 250 and 275 g (2 months of age) at time of delivery were individually housed in standard cages under a 12/12 h light/dark cycle with *ad libitum* access to food and water.

Pregnant female (n=13, three month-old, 240-260g body weight on delivery) Sprague–Dawley rats (OFA, Charles Rivers, France) were individually housed in plastic breeding cages under standard laboratory conditions. After birth, only litters of 8-11 pups with approximately equal sex ratios were retained for the study. The litters were raised by their biological mothers until weaning (21 days after birth). After weaning, only the male progeny were kept, and animals were randomly assigned to the different experimental groups.

All experiments were performed in accordance with the recommendations of the European Union (2010/63/UE) and were approved by the ethical committee of the University of Bordeaux (#Dir1367, #17467, #22698).

Plasmids and retroviruses

The Gs DREADD was cloned by PCR using pcDNA5/FRT-HA-rM3D(Gs) (Addgene #45549; ²⁴) as a template (See table S2 for PCR primers) and then inserted into the BamHI site of a CAG-IRES-GFP retroviral backbone²⁵. The resulting construct CAG-Gs-IRES-GFP was sequencing using specific primers (table S2) and is named Gs-GFP-RV in the text. The control construct had the same viral backbone without the insert (GFP-RV in the text).

High titers of retroviruses were prepared with a human 293-derived retroviral packaging cell line (293GPG)²⁶, kindly provided by Dr Dieter Chichung Lie (University of Erlangen-Nuremberg). Virus-containing supernatant was harvested three days after transfection with Lipofectamine 2000 (Invitrogen, Oregon, US. #11668-019)). This supernatant was then cleared from cell debris by centrifugation at 2191 g for 15 min and filtered through a 0.45 µm filter (Millipore, Massachusetts, US). Viruses were concentrated by two rounds of centrifugation (respectively 46000 g and 67629 g, 1h each) and resuspended in PBS.

Retroviral injections

Adult rats were anaesthetized with 3% isoflurane and placed in the stereotaxic frame, where they were maintained on 2% isoflurane for the duration of the surgery. Analgesia was provided by a subcutaneous injection of Metacam (1mg/Kg). Retroviruses were stereotaxically injected (2 μ L per injection site at 0.3 μ L/min) into the dentate gyrus of adult rats with a microcapillary pipette connected to a micro-syringe pump (KDScientific SPLG130) attached to the stereotaxic frame. To check the efficiency of the virus *in vivo* and the effect on the survival of new neurons, two bilateral injections were made into the Hilus of the dorsal hippocampus through stereotaxic surgery coordinates from Bregma (-3.2mm posterior, $\pm$ 1.6mm lateral, -4.2 ventral). Four bilateral injections were made for behavioral and electrophysiological experiments (-3.2mm posterior, $\pm$ 1.6mm lateral, -4.2 ventral; and -3.8 mm posterior, $\pm$ 1.8mm lateral, -4.2mm ventral).

Rats from postnatal day 3 were anaesthetized on ice and place on a neonatal rat adaptor in the stereotaxic frame. They were maintained on ice during the surgery. Retroviruses were bilaterally injected (1 μ L per injection site at 0.3 μ L/min) into the dentate gyrus of with a microcapillary pipette (from Bregma: -1.2mm posterior, $\pm$ 1.3mm lateral, -2.6 ventral). At the end of the experiment, only rats with labeled-cells in both hemispheres were kept in the analysis.

BrdU injection

BrdU (5-bromo-2'-deoxyuridine, Sigma-Aldrich, Missouri, US) was dissolved in a Phosphate Buffer (pH 8.4) and rats received one injection (100 mg/kg) intra-peritoneally (ip).

Water maze procedures

The apparatus consisted of a circular plastic swimming pool (180 cm diameter, 60 cm height) that was filled with water (20 $\pm$ 1°C), rendered opaque by the addition of a white cosmetic adjuvant. Two days before training, the animals were habituated to the pool for one minute. During training, animals were required to locate a submerged platform (16 cm diameter) hidden 1.5 cm under the surface of the water in a fixed location, using the spatial cues available within the room. All rats were trained for four trials per day (90 s with an inter-trial interval of 30 s, with release from one of three starting points selected

in a pseudorandom sequence each day) during 6 days. If an animal failed to locate the platform itself, it was placed on the platform by the experimenter at the end of the trial. The time to reach the platform was recorded with a video camera that was fixed to the ceiling of the room and connected to a computerized tracking system (Videotrack, Viewpoint, Lyon, France) located in an adjacent room.

Four weeks after learning, rats were submitted to a retention test in the water maze. During the test, rats were put in the water maze for 60 s in the absence of the platform. Performances were assessed using several parameters: the amount of time spent in and number of entries (annulus crossing) into each zone. Zones were defined as an ideal circle (30 cm diameter) located at the original platform location (Target zone; T) and the three equivalent areas in each of the other quadrants (other zones; O). The grouped heat-maps were created with Ethovision XT 10, Noldus (Nantes, France). The color of a pixel represents the average proportion of a track that is found at that location.

Contextual fear conditioning

The fear conditioning chamber consisted of a squared conditioning chamber (30 cm X 30 cm X 40 cm) of a brightness of 10 lux, containing a stainless steel grid floor (Imetronic, Marcheprie, France). Rats were habituated to handling for 3 days before conditioning. On conditioning day, rats were placed in the chamber for 2 min for habituation. Then they received 2 foot shocks (0.8 mA each, 1sec) 60 sec apart (wait 1) and they were left undisturbed for another 60 sec period (wait 2). Chambers were cleaned with 70% ethanol between each rat. On testing day, rats were placed in the chamber for 3 min. Behavioral data were automatically collected using infrared beams spaced 1 cm apart in the x and y planes, located at the floor of the chambers. Freezing behavior was recorded following the cessation of movement for at least 2 sec.

CNO delivery

The DREADD ligand CNO (Clozapin-n-Oxyde, Enzo Life Sciences, Lyon, France #BML-NS105) was dissolved in a saline solution and delivered via one ip injection of 1mg/kg in rats 60 min before the test.

Immunohistochemistry and analysis

Animals were perfused transcardially with a phosphate-buffered solution of 4% paraformaldehyde. After one week of fixation, brains were cut with a vibratome. Free-floating 50 µm thick sections were processed according to a standard immunohistochemical procedure to visualize GFP (Chicken primary antibody, 1:2,000, Abcam, Cambridge, UK. #Ab13970), BrdU (Mouse primary antibody, 1:200, Dako Agilent, Santa Clara, US. #M0744), and Zif268 (Rabbit primary antibody, 1:500, Santa Cruz Biotechnology, Santa Cruz, US. #SC-189) on alternate 1-in-10 sections.

GFP positive cells throughout the entire dentate gyrus were revealed using the biotin-streptavidin technique (ABC kit, Vector Labs, Peterborough, UK #PK-4000) and 3,3'-diaminobenzidine as a chromogen with a biotinylated goat anti chicken antibody (1:500, Jackson ImmunoResearch, Cambridgeshire, UK. #103-065-155).

GFP-IR cells were counted under a 100x microscope objective throughout the entire septo-temporal axis of the granule and subgranular layers of the dentate gyrus (DG). The total number of cells was estimated using the optical fractionator method, and the resulting numbers were tallied and multiplied by the inverse of the sections sampling fraction ($1/ssf_{10}$).

BrdU-positive cells throughout the entire granular layer of the supragranular and infragranular blades of the DG were revealed using the biotin-streptavidin technique with a horse anti-mouse antibody (1:200, Vector Labs, Peterborough, UK. #BA-2001). The total number of cells was counted under a 100x microscope objective throughout the entire left septo-temporal axis of the granule and subgranular layers of the DG as previously described²⁴. The total number of cells was estimated using the optical fractionator method.

Activation of GFP-IR neurons was examined by immunohistofluorescence. GFP was visualized with an Alexa-488 goat anti-chicken antibody (1:1,000, Invitrogen, Oregon, US. #A-11039). In the same sections, Zif268⁺ cells were visualized with an Alexa-568 goat anti-rabbit antibody (1:1,000 Invitrogen, Oregon, US. #A-11011). Double labeling was determined by using a SPE confocal system with a plane apochromatic 63X oil lens (digital zoom of 2). The percentage of GFP cells expressing Zif268 was calculated as follows: $(\text{Nb of GFP}^+\text{-Zif268}^+ \text{ cells}) / (\text{Nb of GFP}^+\text{-Zif268}^- \text{ cells} + \text{Nb of GFP}^+\text{-Zif268}^+ \text{ cells}) \times$

100. For the representative image of Gs-retrovirus GFP fluorescence, nuclei in the dentate gyrus were revealed using DAPI (1:10,000, Invitrogen, Oregon, US. #P36931). Mosaic pictures were taken using a SP8 confocal system with a 20X multi-immersion lens (digital zoom of 1.2).

Electrophysiological recordings

Infected animals were deeply anesthetized (167mg/Kg ketamine and 16,7mg/Kg xylazine) and sacrificed. Dissected brain was immediately immersed in ice-cold oxygenated cutting solution (in mM: 180 Sucrose, 26 NaHCO₃, 11 Glucose, 2.5 KCl, 1.25 NaH₂PO₄, 12 MgSO₄, 0.2 CaCl₂, saturated with 95% O₂-5% CO₂). 350 μ m slices were obtained using a vibratome (VT1200S Leica, Germany) and transferred into a 34°C bath of oxygenated aCSF (in mM: 123 NaCl, 26 NaHCO₃, 11 Glucose, 2.5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂; osmolarity 310 mOsm/l, pH 7.4) for 30 minutes and then cooled down progressively till room temperature (RT; 23-25°C) in oxygenated aCSF. After a 45 min recovery period at RT, slices were anchored with platinum wire at the bottom of the recording chamber and continuously bathed in oxygenated aCSF (RT; 2ml/min) during recording.

Infected newborn granular cells were identified using GFP with a fluorescence/infrared light (pE-2 CoolLED excitation system, UK). Neuron action potential firing was monitored in whole-cell current-clamp recording configuration. Patch electrodes were pulled (micropipette puller P-97, Sutter instrument, USA) from borosilicate glass (O.D. 1.5 mm, I.D. 0.86 mm, Sutter Instrument) to a resistance of 2-4 M Ω . The pipette internal solution contained [in mM: 125 potassium gluconate, 5 KCl, 10 Hepes, 0.6 EGTA, 2 MgCl₂, 7 Phosphocreatine, 3 adenosine-5'-triphosphate (magnesium salt), 0.3 guanosine-5'-triphosphate (sodium salt) (pH adjusted to 7.25 with KOH; osmolarity 300 mOsm/l adjusted with d-Mannitol)] as well as biocytin 0.4% (a liquid junction potential of -14.8mV was corrected for in the data and statistics).

CNO (10 μ M in aCSF) was fast perfused close to the recording cell for 30 seconds then immediately washed out. Electrophysiological data were recorded using a Multiclamp 700B amplifier (Molecular devices, UK), low-pass filtered at 4 kHz and digitized at 10Hz (current clamp) or 4 Hz (voltage clamp) (Digidata 1440A, Molecular devices, UK). Signals were analyzed offline (Clampfit software, pClamp 10, Molecular devices, UK). For

statistical analysis, “Vehicle” data were collected during the last 60 seconds before CNO perfusion, then “CNO” data were collected after 45 seconds of CNO treatment.

Statistical analysis

The data (mean±SEM) were analyzed using the Student t-test (two-tailed) and two ways ANOVA which was followed by the Tukey’ comparison test when necessary. All analyses were carried out using the software GraphPad Prisms 6 and 8.

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Author contributions.

ML designed and performed the experiments and analyzed the data. PM performed the experiments. EP designed the retroviruses. FF produced the retroviruses. GT, WM and VC performed the electrophysiological experiments. DC supervised electrophysiological experiments and revised the paper. GF designed the experiments and revised the paper. DNA conceived experiments and wrote the paper. ST conceived and designed the

experiments, performed experiments, analyzed the data and wrote the paper. All the authors edited and approved the final version of the manuscript.

Competing Interests: The authors declare no competing interests.

Data availability

All data supporting the findings of this study are provided within the paper and its supplementary information. A source data file is provided with this paper. The CAG-Gs-IRES-GFP retroviral construct is available upon request to the authors after MTA approval. All additional information will be made available upon reasonable request to the authors.

Figure legend

Figure 1. Long-term memory retention four weeks after learning. **a** – Two-month-old adult rats were trained in the WM for 6 days. One group of rats (n=8) was submitted to a probe test 48h after training and another group of rats (n=9) was tested 4 weeks after training. **b** – Latency to reach the platform during learning and to first cross the position of the platform during the tests. There was no difference between groups at learning and the latency to reach the platform position was similar at 48h or 4 weeks after training. **c** – Number of annulus crossings (zone entries), animals show a significant preference for the target zone (**T**) compared to the other zones (**O**) when tested 48h after training whereas no difference was observed at the 4w test (zone X group interaction $F(1,15)=7.732$; $p=0.014$; zone effect $F(1,15)=11.66$; $p=0.0038$; group effect $F(1,15)=5.233$; $p=0.037$) **d** – Percentage of time spent in the target zone compared to that of the other zones. Rats that were tested 48h after training spent significantly more time in the target zone compared to the other zones and the amount of time was

higher than that of chance level. No difference was observed when animals were tested 4 weeks after training (zone X group interaction $F(1,15)=7.145$; $p=0.017$; zone effect $F(1,15)=12.60$; $p=0.0029$; group effect $F(1,15)=2.762$; $p=0.1173$) (** $p<0.01$; # $p<0.05$ compared to chance level). **e**- Density plot for grouped data: The color level represents the lowest to the highest location frequency in pixels. All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 2. Stimulation of Gs-GFP-RV is efficient in vitro and in vivo. a – GFP

fluorescence in DG labeled neurons 6 weeks post-injection of the Gs-GFP-RV (scale: 100 μ m). **b** – Injection of retroviruses does not impact the survival of new neurons 6 weeks post-injection ($t_6=0,07$, $P=0,9451$; unpaired t -test; $n=3$ GFP- and $n=5$ Gs-GFP-rats). **c** – GFP and Gs-GFP retroviruses were injected in the left and right DG, respectively, of adult rats. Six weeks later, rats were injected with CNO (IP, 1mg/Kg) and sacrificed 2h later. **d** - CNO Injection strongly enhances Gs-GFP cell activation in vivo compared to GFP cells. ($t_3=54,90$; *** $P<0,0001$; Paired t -test; $n=4$ rats). *Right:* illustration of a Gs-GFP cell (green) expressing Zif268 (red) (scale: 20 μ m). **e** – Scheme for whole-cell recording from Gs-GFP or GFP cells in the DG. **f** - Representative traces of 10 μ M CNO perfusion effect onto cellular activity. CNO is inert in GFP cells, but quickly and reversibly enhances Gs-GFP cell activity. **g** - CNO depolarizes Gs-GFP cells and increases their action potential firing. Blue lines represent data from individual cells, black lines are the mean of the blue lines. (Paired t -tests, Vehicle vs CNO treated rats. Resting potential ($t_7=3,22$; * $P=0,0146$); Action potential firing ($t_7=3,51$; ** $P=0,0098$; $n=2$ rats and 8 cells)). **h** – CNO has no effect on resting potential and action potential firing frequency of GFP cells. Grey lines represent data from individual cells, black lines are the mean of the grey lines. (Paired t -tests, Vehicle vs CNO applied for 30s, treated rats.

Resting potential ($t_3=1,016$; $P=0,3845$) Action potential firing ($t_3=0,42$; $P=0,7029$; $n=3$ rats and 4 cells)). All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 3. Pharmacological stimulation, during remote retention test, of adult-born neurons that were immature at the time of learning enhances memory accuracy. a

- Timeline of behavioral procedure with water maze training 1 week after retrovirus injections (Gs-GFP-RV rats, $n=12$; GFP-RV rats, $n=14$) and retention test four weeks after training. CNO was injected 1h before the test. **b** - Latency to find the hidden platform: For all groups latency decreases over time during training and no difference was observed at the retention test. **c** - Number of annulus crossings during test: Gs-GFP-RV rats entered more in the target zone than in the other zones whereas no difference was observed in GFP rats (zone X group interaction $F(1,24)=6.9$; $p=0.0148$; zone effect $F(1,24)=10.26$; $p=0.0038$; group effect $F(1,24)=8.374$; $p=0.008$). **d** - Gs-GFP-RV rats spend more time in the target zone compared to GFP-RV rats. For Gs-GFP rats, the time spent was significantly higher in the target zone compared to that in the other zone and higher than the chance level (zone X group interaction $F(1,24)=4.326$; $p=0.0484$; zone effect $F(1,24)=4.458$; $p=0.0453$; group effect $F(1,24)=1.922$; $p=0.1784$). **e** - Density plot for grouped data: The black circle represents the position of the platform during training. The color level represents the lowest to the highest location frequency in pixels. **f**- Number of GFP-IR in the DG on alternate 1-in-10 50 μ m sections. The retrovirus infection spreads at least 4mm across the septotemporal dentate gyrus axis. (* $p<0.05$; ## $p<0.01$ compared to chance level). All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 4. Pharmacological stimulation, during remote retention test, of adult-born neurons that were mature at the time of learning enhances memory accuracy. a -

Timeline of behavioral procedure with water maze training 6 weeks after retrovirus injections (Gs-GFP-RV rats, n=10; GFP-RV rats, n= 9) and retention test four weeks after training. CNO was injected 1h before the test. **b** – Latency to find the hidden platform: For all groups latency decreases over time during training and no difference was observed at the retention test. **c** – Number of annulus crossings during test: Gs-GFP-RV rats entered more in the target zone than in the other zones whereas no difference was observed in GFP rats (zone effect $F(1,17)=14.63$; $p=0.0014$). **d** – For Gs-GFP rats, the time spent was significantly higher in the target zone compared to that in the other zone and higher than the chance level (zone effect $F(1,17)=16.87$; $p=0.0007$). **e** - Density plot for grouped data: The black circle represents the position of the platform during training. The color level represents the lowest to the highest location frequency in pixels. **f**– Number of GFP-IR in the DG on alternate 1-in-10 50 μ m sections. The retrovirus infection spreads at least 4mm across the septotemporal dentate gyrus axis. (* $p<0.05$; ## $p<0.01$ compared to chance level). All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 5. Pharmacological stimulation, during remote retention test, of neurons generated during development has no effect on memory. a -

Timeline of behavioral procedure with retrovirus injections performed 3 days after birth (Gs-GFP-RV rats, n=12; GFP-RV rats, n= 13), water maze training 8 weeks after injections and test four weeks after training. CNO was injected 1h before the test. **b** – Latency to find the hidden platform: For all groups latency decreases over time during training and no difference was observed at the retention test. **c** – Number of annulus crossings during test: there

was no difference between groups, with no preference for the TZ. **d** –Time spent in TZ was not different than the one spent in the other zones for both groups. All time were at chance level **e** - Density plot for grouped data: The black circle represents the position of the platform during training. The color level represents the lowest to the highest location frequency in pixels. **f**– Number of GFP-IR in the DG on alternate 1-in-10 50µm sections. The retrovirus infection spreads at least 4mm across the septotemporal dentate gyrus axis. All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 6. Pharmacological stimulation, during remote retention test, of neurons that were immature during CFC improve memory retention. **a** - Timeline of behavioral procedure with contextual fear conditioning (CFC) 1 week after retrovirus injections (Gs-GFP-RV rats, n=9; GFP-RV rats, n= 8) and test five weeks after training. CNO was injected 1h before the test. **b**- Design of conditioning and test protocol. **c**- Percentage of freezing during conditioning: there was no freezing difference between the groups. **d**- Percentage of freezing during test: Gs-GFP rats froze significantly more than control GFP animals ($t(15)=2.298$; $p=0.0363$). **e**– Number of GFP-IR in the DG on alternate 1-in-10 50µm sections. The retrovirus infection spreads at least 4mm across the septotemporal dentate gyrus axis. (* $p<0.05$). **f**- GFP-labeled neurons in DG 6 weeks post-injection of the Gs-GFP-RV (scale: 100µm). All data shown are mean $\pm$ s.e.m. For statistical details, see table S1.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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