cFos Ensembles in the Dentate Gyrus Rapidly Segregate Over Time and do not Form a Stable Map of Space

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cFos ensembles in the dentate gyrus rapidly segregate over time and do not form a stable map of space

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Abstract

Mice require several days of training to master the water maze, a spatial memory task for rodents. The hippocampus plays a key role in the formation of spatial and episodic memories, a process that involves the activation of immediate-early genes such as cFos. We trained cFos-reporter mice in the water maze, expecting that consistent spatial behavior would be reflected by consistent cFos patterns across training episodes. Even after extensive training, however, different sets of dentate gyrus (DG) granule cells were activated every day. Suppressing activity in the original encoding ensemble helped mice to learn a novel platform position (reversal learning). Our results suggest that even in a constant environment, cFos+ ensembles in the dorsal DG segregate as a function of time, but become partially reactivated when animals try to access memories of past events.

1 Introduction

2 An accurate memory of past events and their outcomes is of high adaptive value for any
3 animal, as past experience is a useful guide for decision making. The hippocampus is famous
4 for its processing of spatial information^{1,2}, but it also processes other types of sensory input
5 and is capable of grouping events together in time to form episodic memories^{3–5}. How time
6 is represented in the hippocampus is still incompletely understood. Many genes affecting
7 synaptic plasticity undergo pronounced circadian oscillations, changing the rules of synaptic
8 plasticity depending on the time of day⁶. Theoretical and empirical work suggests that the
9 dentate gyrus (DG) actively reduces the overlap of activity patterns coming from entorhinal
10 cortex^{7–9}. In vivo electrophysiology and calcium imaging experiments revealed that the
11 majority of GCs are silent, and of the active cells, just a small fraction show spatial tuning
12 (“place cells”)^{10–16}. Spatially-tuned GCs provide a stable, albeit coarse representation of the
13 global environment across time^{10,13,16}. These findings highlight an obvious design conflict:
14 While a perfect ‘episode encoder’ should avoid using the same neurons on consecutive days,
15 accurate place coding, at least in the sense of labeled lines, requires consistent activation of
16 specific neurons to signal the animal’s position in a given environment. In view of this
17 conundrum, we set out to study the impact of time and space on the activity of granule cells
18 in dorsal DG.

19 To monitor and manipulate neuronal activity in freely behaving animals, reporter mice have
20 been developed that use immediate-early genes^{17,18} (cFos, Arc) to drive the expression of
21 fluorescent proteins and optogenetic actuators¹⁹. Fear conditioning experiments with
22 activity-dependent expression of optogenetic silencing tools suggest that recall of a fearful
23 episode requires reactivation of the original encoding ensemble^{18,20,21}. Vice versa, artificial

reactivation^{20,22–24} of the encoding ensemble has been shown to reinstate a fearful state, suggesting that memories can indeed be activated by specific subsets of hippocampal neurons. In these experiments, freezing was used as a proxy to assess the emotional state of the animal. However, due to the one-dimensional read-out, it is not possible to distinguish the precise recall of the conditioning episode from a general state of heightened anxiety.

We were interested in how the activity of old and new ensembles in DG would affect decision making in situations where the animal has a large number of possible choices. TetTag mice allow comparing activity-dependent expression of cFos at two different time points that can be several days apart^{23,25}. To be able to manipulate activity in freely behaving animals, we injected a virus encoding the chloride-conducting channelrhodopsin iChloC²⁶ for light-controlled neuronal inhibition. We adapted the classic Morris water maze for mice tethered with thin optical fibers, using a helium balloon to compensate for the weight of the implant. As the expression of the inhibitory tool was restricted to the neurons active in the first tagging window, we were able to selectively silence this ensemble during probe trials or reversal training. To our surprise, optogenetic inhibition had either no effect or even improved performance in the water maze. This finding was explained by the low cFos ensemble reactivation (overlap) at different stages of water maze training. Our results strongly support the proposed function of the DG in pattern separation and suggest that under conditions of high interference, reactivation of old ensembles can hinder the formation of new spatial memories.

Results

Stable expression of optogenetic tools in active neurons for 6 days in TetTag mice

To tag neuronal activity at two different time points, we bilaterally injected the DG of TetTag mice with AAV9-TRE-iChloC-mKate (Fig. 1a). Neuronal activity in TetTag mice activates the expression of two transgenes via the cFos promoter: 1) tetracycline transactivator (tTA) and 2) a short half-life, nuclear-localized, enhanced green fluorescent protein (shEGFP). In the absence of doxycycline (off-Dox), tTA binds to the tTA-response element (TRE) from the virally delivered gene, resulting in permanent mKate tagging of neurons active in the off-Dox time window (magenta). Green nuclear fluorescence (shEGFP) identifies neurons that were active in the hours before the animal was sacrificed (Fig. 1b). In calibration experiments on slice cultures from TetTag mice, we found that shEGFP fluorescence peaked 3.5 h after high K^+ stimulation (Extended Data Fig. 1), suggesting that green-labeled neurons were active 2-4 h before sacrificing the animal. Cells that were active off-Dox and again in the hours before euthanasia could be readily identified *ex-vivo* by their magenta (mKate) membrane and green (shEGFP) nucleus, both of which were amplified by indirect immunofluorescence (Fig. 1b).

To evaluate the ensemble size and stability of iChloC-mKate across time, injected TetTag mice were taken off-Dox two weeks after AAV injection. The bedding in the home cage was changed to prevent mice from eating any Dox food crumbs. Twenty-four hours later, mice were either kept undisturbed in their home cage or trained (6 trials, see Methods) in the water maze (WM). Home-caged mice were sacrificed on the same day ($n = 3$ mice) or 1 day later ($n = 4$ mice). WM-trained mice were sacrificed 1 day later ($n = 4$) or 6 days later ($n = 7$

66 mice) (Fig 1 c). To compare cFos tagging efficiencies between virally transfected (iChloC-
67 mKate) and transgenic (shEGFP), mice were trained in the WM on the day of euthanasia
68 (identical number of trials). Mice that were always on-Dox (home caged) showed no mKate
69 expression. Home-caged mice sacrificed on tagging day (0 d) showed significantly fewer
70 mKate+ cells than mice sacrificed on the following day, probably due to the slow maturation
71 of iChloC-mKate. Surprisingly, WM-trained mice showed similar ensemble sizes as home
72 cage-exploration mice, probably because the bedding change triggered cFos expression as
73 seen when novel objects are introduced in their home cage ²⁷. Because WM learning
74 requires more than one training day to develop a consistent allothetic navigation strategy, it
75 was crucial that the cFos neuronal ensemble tagging lasted for at least six days. We found
76 similar ensemble sizes in WM-trained mice that were sacrificed 1 or 6 days later (Fig. 1 d, e).

Figure 1. General experimental design for tagging two c-Fos⁺ neuronal ensembles suitable for WM training. **a**, TetTag mice containing two cFos-driven transgenes: 1) tetracycline trans-activator (tTA) and 2) nuclear-localized, short-life enhanced green fluorescent protein (shEGFP). To tag (and manipulate) cFos⁺ cells, TetTag mice are injected bilaterally in DG with an AAV containing the improved version of a chloride-conducting channel rhodopsin (iChloC) under the control of a tTA response element (TRE). Doxycycline (black triangle) prevents tTA from activating TRE-iChloC-mKate expression. When Dox is removed, active GCs express TRE-iChloC-mKate. shEGFP expression is independent of Dox. **b**, Experiment timeline. TetTag mice were injected with AAVs in DG on-Dox. Two weeks later, Dox was removed for 24 h (Dox-off). Some mice were in their home cage (HC) and sacrificed 24 h after Dox removal (0d, n = 3) or 48 h after Dox removal (1d, n = 3). A different cohort of mice experienced water maze (WM) training off-Dox. After training, mice were put back on Dox and were either sacrificed 48h after Dox removal (1d, n = 4) or 6 days after (6d, n = 7). **c**, Confocal images showing DG. All tagged-GCs were counted within the limits of the GC layer (DAPI). iChloC-mKate expresses on GC membrane (pseudocoloured in magenta) while shEGFP (green) is only in the nuclei. Overlapping cells show magenta membrane and green nuclei. **d**, Confocal image set showing both GCs ensembles for all conditions. Dox prevents iChloC-mKate expression. **e**, Ensemble size (mKate⁺/DAPI and shEGFP⁺/DAPI). mKate⁺ ensemble size is significantly lower in the 0d than 1d (*, p=0.03, Brown-Forsythe ANOVA, Dunnett multiple comparisons 0d vs. 1d). mKate⁺ ensemble size was similar regardless of experience (HC or WM) 1d after Dox removal. mKate ensemble size was not decreased 6 days after tagging. shEGFP ensemble size was similar regardless of experience (HC or WM) or day of sacrifice.

Optogenetic silencing of cFos+ DG ensembles in the water maze

In fear conditioning experiments, cFos+ ensembles are often regarded as the trace of a particular memory¹⁹. To test whether the recall of a spatial memory requires reactivation of the same set of neurons that were cFos+ during the first encoding day, we tagged cFos+ cells in DG with the light-activated chloride channel iChloC²⁶ (n = 16 mice) (Fig. 2a). Compared to light-driven pumps, this channel requires lower light intensities for efficient inhibition²⁸, allowing the inhibition of a larger volume of tissue from a single light fiber. In the absence of light, membrane properties of iChloC-expressing GCs were indistinguishable from those reported in the literature²⁹ (Extended Data Fig. 2). Blue light (473 nm) activated iChloC, preventing action potential generation (Extended Data Fig. 2). For inhibition of tagged GCs in vivo, blue light pulses (1 Hz) were delivered bilaterally through a two-ferrule cannula targeting the hippocampal sulcus directly above DG (Fig. 2a). To aid the swimming of tethered mice, we overcompensated the weight of the implant and the two optical fibers with a helium balloon, resulting in a slight pull-up force of 0.07 N (Fig. 2b). Under these conditions, the head was held above the water surface, and mice swam as fast as in untethered trials (Extended Data Fig. 3). After two training days, mice reached average escape latencies below 30 s (Fig. 2d). On day 3, they were tested for spatial reference memory (no platform, 60 s probe trial (PT)) with and without optogenetic silencing of the original encoding ensemble (i.e., cFos+ cells tagged on day 1). The experiments were designed to have light ON/OFF probe trials in each mouse, counterbalancing to observe potential light effects (and not trial effects). We quantified the time spent close to the target position (annulus = platform diameter $\times$ 3.5 = 35 cm) to the time spent in the corresponding area in the opposite quadrant (Fig. 2e). Optogenetic silencing on day 3 had no effect on WM performance (Fig. 2f). However, mice had not yet developed full spatial accuracy. Therefore,

102 we trained the mice further until they reached asymptotic escape latencies (< 20 s) and then
103 tested again for potential effects of optogenetic inhibition on day 5. In the probe trial, all
104 mice showed a spatially accurate search in the target region (Fig. 2g). To our surprise,
105 optogenetic silencing of cFos+ cells significantly *increased* the time spent in the target region
106 (annulus E). We detected no effects of optogenetic inhibition on swim speed or distance
107 moved (Fig. 2h, figure 3). Apparently, activity in the original encoding ensemble contributed
108 noise rather than useful spatial information and thereby interfered with spatial learning and
109 memory consolidation (Fig. 2g). We confirmed that blue light prevented action potentials in
110 iChloC expressing GCs (Fig. 2i, Extended Data Fig. 3)

Figure 2. Optogenetic silencing of day 1 cFos-tagged DG ensembles improves spatial memory recall in over trained (day 5) mice but not at early training (day 3) phases of the WM. **a**, TetTag mice were injected with AAV-TRE-iChloC-mKate2 and a bilateral fiber-optic cannula was implanted above DG. For optogenetic inhibition, 20ms (1Hz), 473nm light pulses were used. **b**, Setup for optogenetic stimulation in the water maze, using calibrated pull-up by helium balloon. **c**, Experimental design. Mice (n = 16) were trained in the WM with a platform in quadrant E, on day 1, off-Dox and put back on-Dox afterward (tagging day). On day 2, mice were trained again with tether, but no optogenetic manipulation was done (early training). Before day 3 training, mice were tethered and were tested twice (probe trial light ON/OFF) for reference spatial memory. Mice were trained untethered 4. On day 5, mice had 2 probe trials with and without light. **d**, Schematic representation virtual divisions of the WM. **e**, Escape latency at different stages of training. Mice reach asymptotic performance on day 5. **f**, Counterbalanced (light OFF/ON) probe trials on day 3. Mice show a significant preference for annulus E over W (****, $p < 0.0001$, effect of annulus position by mixed 2-way-ANOVA) but no interaction between light and time in the annulus ($p = 0.47$, matched, 2-way-ANOVA). **g**, Counterbalanced (light OFF/ON) probe trials on day 5. Mice show a significant preference for annulus E over W (****, $p < 0.0001$, effect of annulus position by mixed 2-way-ANOVA) and light significantly increases the spent time in annulus E (* $p = 0.04$, Bonferroni test after mixed 2-way ANOVA: annulus E, light OFF vs. ON). **h**, Swim speed and distance swum are not affected by light on day 5. **i**, Hippocampal slice cultures were used to test the effect of blue light on action potential formation in GCs expressing iChloC.

Optogenetic silencing during reversal training improves reversal learning

To test the memory interference hypothesis, we evaluated how silencing of the “old” (day 1) representation affected the acquisition of a new memory. The same cohort (n = 16) of AAV-injected TetTag mice (Fig. 2) was trained on day 6, but we changed the platform position to the opposite quadrant (W) (reversal training, RT, Fig. 3a, c). Mice were separated into two groups with similar performance in the probe trial (light OFF) on day 5 (Extended Data Fig. 3). All mice were tethered; some received light during reversal training (RT-silenced, n = 9 mice) and some did not (RT-control, n = 7 mice). Both groups rapidly learned the new escape location, showing a significant decrease in their escape latencies during the training trials (Fig. 3b). We noticed, however, a difference in spatial precision: In the last training trial, silenced mice had developed a clear memory of the new platform location while control mice had not significantly improved compared to the first reversal training trial (Fig. 3d). Thus, silencing neurons associated with the early acquisition (day 1) of the experimental arena and first platform position improved the learning of a new platform position. After the reversal training trials, reference memory was tested in a tethered probe trial without optogenetic silencing. Reversal learning was incomplete, as control animals still had a significant preference for the old platform position (E). In contrast, mice that were trained under inhibition showed a better memory of the new platform position, visiting both old and new platform positions with equal frequency (Fig. 6e, f). Analysis of the swimming patterns of individual animals over time revealed that the behavioral difference was most obvious in the first 30 s of the probe trial: Mice that were trained under inhibition spent significantly more time around the new platform position compared to controls (Fig. 3f, g). Thus, optogenetic suppression of the DG ensemble that was active during early training of the first platform position significantly improved reversal learning. Finally, to investigate the effect of

136 optogenetic inhibition on cFos expression, we processed all brains for immuno-enhanced
137 confocal imaging. To estimate the fraction of active neurons in dorsal DG (ensemble size),
138 we normalized the number of cFos+ neurons by the total number of granule cells in each
139 brain section (6 sections per animal), estimated from DAPI staining. The size of cFos
140 ensembles was similar on day 1 and day 6, suggesting the same number of GCs were
141 engaged on both days (Fig. 3g). The cellular overlap between ensembles was lower than
142 chance in all mice, suggesting that a different set of GCs was used to represent the events of
143 day 1 and day 6, despite the environmental familiarity. Mice from the silenced group
144 showed significantly lower overlap than the control group, demonstrating successful
145 optogenetic inhibition of the day 1 ensemble on day 6 (Fig. 3h). These results suggest that
146 activity in the originally active ensemble (day 1 cFos+ neurons) interferes with the rapid
147 acquisition of new spatial information several days later. To dissect if the low overlap was
148 due to the temporal distance between tagging days or driven by environmental factors, we
149 decided to evaluate the effects of time, environmental changes, and learning in separate
150 experiments.

Figure 3. Optogenetic silencing of day 1 cFos-tagged DG ensembles improves reversal training acquisition and recall. **a**, Experimental design. The same cohort of mice from Fig. 2 was separated into two groups with similar performances during the last light OFF PT on day 5. Both groups were tethered and underwent four training trials with the platform in annulus W (reversal training, RT). RT-silenced mice received 20 ms, 473 nm light pulses during the whole length of each learning trial. 1.5h after RT, mice were sacrificed for cFos ensemble immunofluorescence assay. **b**, Escape latency of each group (only trial 1 and 4 shown). **c**, Schematic representation of WM tank virtual divisions. **d**, Shift in search behavior over RT trials on day 6. Heatmaps showing average mouse position for the RT-C (top row) and RT-S (bottom row) groups. Mean minimal distance to the new platform position (W) over the four RT trials. The RT-S group, but not the RT-C group, swim closer to the new platform position during trial four compared to trial one (***, $p=0.0004$ trial; Tukey's test after mixed 2-way ANOVA). **e**, PT 60s, no platform, no light, tethered. Both groups showed similar spatial memory retrieval but mice in the RT-S group showed no longer preference for annulus E over W while RT-C does (*, $p=0.02$ trial; Tukey's test after mixed 2-way ANOVA). **f**, Raster plot of behavior per individual mice during the PT (60 s). Time spent in annuli (W shown in pink, E shown in blue) gray indicates time spent outside annuli. Numbers on the right indicate platform crossings. PT divided in 30 s time bins shows mice in the RT-S group search in both annuli while mice in the RT-C show a significant preference for annulus E in the first 30s of the PT (** $p=0.009$, test 0-30s vs. 30-60s, Bonferroni's multiple comparisons after mixed 2-way ANOVA). **g**, cFos-tagged cells from total of DAPI cells from DG GC layer. Both groups show similar ensemble sizes for day 1 (mKate+) and day 6 (shEGFP+). **h**, overlap/chance was significantly lower in the RT-S group (*, $p=0.03$, unpaired t-test). **i**, Venn diagram shows ensemble overlap is minimal in both groups.

In a constant environment, GC ensemble selection is a function of time.

In vivo recordings of GCs indicate that the ones that encode spatial information show low remapping across time in the same environment and high remapping in different environments^{11,13,15,30}. Our expectation was, therefore, that cFos+ neuronal ensembles would remain stable over time as long as the setting is not changed. To test this hypothesis, we used TetTag mice injected with AAV9-TRE-iChloC-mKate2 which were kept in their home cage throughout the experiment. We sacrificed the animals at 3 different time points after the end of the off-Dox period: Immediately ($\Delta 0$ h, $n = 3$), after 6 h ($\Delta 6$ h, $n = 3$), and after 24 h ($\Delta 24$ h $n = 4$). In the $\Delta 0$ h group, 24% of tagged GCs were double-positive. The smaller mKate+ ensemble size in the $\Delta 0$ h and $\Delta 6$ h groups compared to the $\Delta 24$ h group, in contrast to the consistent shEGFP+ ensemble size in all groups, confirmed that the TRE-driven construct took at least 24 h to reach full expression (Fig. 4a, c and Fig. 1d, e). The number of double-positive cells was similar in the $\Delta 6$ h and $\Delta 0$ h groups, indicating that active neuronal ensembles are stable over short (< 6 h) periods. In contrast, the $\Delta 24$ h group showed a surprisingly low number of double-positive cells (Fig. 4c). To compensate for the different sizes of the first encoding ensemble at the different time points, we compared the actual number of double-positive cells to the expected number if the second set of neurons had been randomly selected (chance level, Fig. 4d). The degree of overlap was much higher than expected by chance for the 0 h and 6 h groups, suggesting that the same neuronal ensemble was active at two time points within a short time window. After 24 h, the overlap between the ensembles had fallen slightly below chance level, suggesting that even in a constant environment (i.e., the home cage), a new set of GCs is used for memory encoding on consecutive days (Fig. 4d). As even after 5 days of water maze training, the overlap was not

further reduced (Fig. 3h), we suggest that ensemble reactivation may be a sigmoidal function of time, dropping to chance levels after ~12 h (Fig. 4e).

In addition to counting shEGFP+ neurons, we analyzed the intensity of green fluorescence in these neurons to estimate the degree of activation. We found the highest expression levels in double-positive cells sacrificed right at the end of the off-Dox labeling window ($\Delta 0$ h, Fig. 4f). This is not surprising: As the labeling windows overlapped for several hours, selecting mKate-positive GCs biased the sample towards highly active neurons with high cFos levels. After 6 h on-Dox, shEGFP expression levels in double-positive GCs was already reduced, and after 24 h on-Dox, the difference between shEGFP expression in double-positive GCs and in GCs expressing only shEGFP had completely disappeared. This analysis of expression levels confirmed our conclusion from the overlap analysis, that individual GCs do not maintain high cFos levels for more than a few hours and are rarely reactivated, causing the global pattern of cFos expression in DG to change over time.

In GCs expressing only shEGFP (the large majority of shEGFP+ cells), expression levels were independent of the time point of sacrifice, as expected (Fig. 4f). Together with the constant size of the shEGFP ensemble, this indicates that Dox withdrawal and bedding change had little or no effect on the overall activity in DG.

Figure 4. In the familiar home cage (HC), cFos+ GC ensembles strongly segregate over time. **a**, Experimental design: TetTag mice were injected with AAV9-TRE-iChloC-mKate. Dox was removed (off) for 24h and mice were sacrificed immediately after ($\Delta 0h$ group, $n = 3$). Dox was given back (on) and mice were either sacrificed 6 ($\Delta 6h$ group, $n = 3$) or 24h after ($\Delta 24h$ group, $n = 4$). **b**, Representative confocal images of cFos-tagged GCs. cFos-tagged cells during the off-Dox period express mKate (pseudo-colored in magenta). Transient tagged cFos GCs from hours prior to sacrifice express shEGFP (green). **c**, Venn diagram of tagged cells show ensemble overlap. **d**, Overlap/chance is similar in the $\Delta 0h$ and $\Delta 6h$ groups (ns, $p = 0.11$, unpaired t-test). Both the $\Delta 0h$ and $\Delta 6h$ groups have significantly higher overlap/chance than the $\Delta 24h$ group (****, $p < 0.0001$, unpaired t-test). **e**, Overlap/chance follows a sigmoidal function over time. **f**, Double-labeled GCs (mKate+/shEGFP+) expressed shEGFP significantly stronger immediately after the bedding change ($\Delta 0h$) compared to 6 h later (*, $p = 0.01$), or 24 h later (****, $p = 0.01$). In GCs expressing only shEGFP, not mKate, expression levels were identical at all time points (two-way-ANOVA, Tukey).

cFos ensembles get minimally reactivated during spatial learning

The unexpectedly low overlap of the cFos+ ensembles in a constant environment could have been caused by the fact that hippocampal circuitry is barely engaged when mice are in the home cage. Thus, we next tested whether the amount of overlap increases when animals explore a new environment or develop a spatial navigation strategy. Given the strong effect that time has on the reactivation of cFos ensembles, we kept a consistent 24 h interval between cFos-tagging events. AAV-injected mice were taken off-Dox and trained in the WM with a platform in the E quadrant. After training, mice were fed again with Dox and, 24 h later, they either were trained in the WM with the same platform position (early training, ET) or allowed to explore an unfamiliar open field (novel environment, NE, Fig 5a). To maximize novelty, the NE group was tested in a different experimental room and handled by a different researcher. On day 1, mice in both groups showed similar escape latencies in the water maze. On day 2, only mice from the ET group were further trained in the WM, showing a further significant decrease in escape latency (trial 1 vs trail 8, Fig. 5b). Reference memory was tested on both days in probe trials without the platform (Fig. 5c). On day 1, mice did not show any significant preference for the platform position (annulus E) when compared to the opposite side of the maze (annulus W). At the end of training day 2, the ET group had learned the task as indicated by a significant preference for the target annulus (Fig. 5c). Three hours after the last trial, mice were sacrificed for cFos ensemble analysis. As in the previously described experiments, we counted tagged cells and compared the number of double-positive neurons to the expected overlap for randomly selected neurons. cFos+ ensemble size was similar in both groups for both days (Fig. 5d, Extended Data Figure 4).

Figure 5. cFos+ GC ensembles get partially reactivated during early training phases of WM but segregate when mice visit an unfamiliar environment. **a**, Experimental design. TetTag mice were injected with AAV-TRE-iChloC-mKate in DG and trained while Dox (-) in the WM (platform in E). Mice were then put back on Dox (+) and 24h later, they were either trained again in the WM (early training, ET) or visited an open field arena in a different room for 20 mins (novel environment, NE). Mice were sacrificed 3h after the last trial for cFos-tagged GCs immunofluorescence assay. **b**, Escape latencies from the first and last trial of day 1, show similar learning in both groups. Mice in the ET group significantly reduced their escape latency from day 1 to day 2 (*, $p < 0.05$, repeated measures one-way-ANOVA test). **c**, Reference memory PT, no platform. Heatmaps from each day per group. Representative OF track of one mouse from the NE group on day 2. **d**, Quantification of time in each annuli during PT. On day 1, mice show similar spatial reference memory performance in both groups but no significant preference for annulus E. On day 2, mice from the ET group show a significant preference for annulus E (*, $p < 0.05$, RM-two-way-ANOVA). **e**, Representative confocal images of DG GC layer. ET, top; NE bottom. Arrows indicate GCs with cFos on both days. **f**, Ensembles size. Both groups show similar ensembles size for both days. **g**, Overlap/chance is significantly higher in the ET group than the NE or HC $\Delta 24h$ group from Fig. 4. (*, $p < 0.05$, t test)

Overlap/chance was significantly higher in the ET group than both the NE and 24 h HC group, suggesting that mice do reactivate yesterday's GCs during multi-day spatial learning (Fig. 5d, e). Exposure to a novel environment without any link to the previously trained water maze

task did not drive ensemble reactivation in DG. The number of cFos⁺ neurons labeled on day 2 (shEGFP) did not differ between the three groups. However, within the set of shEGFP-expressing neurons, expression levels were significantly higher in the ET group compared to the NE and home cage groups (Extended Data Figure 4). These results suggest that spatial navigation, a hippocampus-dependent task, activates GCs more strongly than the exploration of a new environment, even though the total number of cFos⁺ neurons was constant under all conditions. Although significantly higher than the NE and $\Delta 24h$ groups, reactivation levels in the ET group were still low compared to the $\Delta 0h$ or $\Delta 6h$ home cage groups.

Well-trained animals facing a novel challenge

Comparing day 1 and day 2 of WM training, we found little overlap between cFos ensembles, but the behavioral performance on day 1 was also very poor. What about two consecutive training days in mice that are already experts at finding the hidden platform? We used AAV-injected mice that were kept on-Dox during the first 4 training days, were trained off-Dox on day 5, and put back on Dox immediately after training. On day 6, mice were either trained again (overtraining, OT) or trained with the platform in the opposite quadrant (W) to test the spatial interference (reversal training, RT, Fig. 6a). Both groups showed excellent performance on day 5, the first tagging day. Mice from both groups did not further improve during this day, indicating asymptotic learning (escape latency < 15 s). On day 6, mice of the OT group maintained their performance, and RT mice improved their performance during the 4 reversal training trials (Fig. 6b). Spatial reference memory was tested in a probe trial at the end of each training day. On day 5, mice in both groups showed spatially precise search behavior, swimming in the proximity of the platform position

243 (annulus E). On day 6, the OT group preferentially searched at annulus E while the RT group
244 searched at both the old (annulus E) and new (annulus W) platform positions (Fig. 6c, e). The
245 restricted spatial search was evident in the heat maps (averaged over all animals) and could
246 also be seen in the behavior of each individual mouse during the probe trials of day 5 and
247 day 6 (Fig. 6d). Mice were sacrificed 3 h after the last trial and brains were processed for
248 cFos mapping. Consistent with previous experiments, we observed similar ensemble sizes
249 from both days in both groups (Extended Data Figure 4). Contrary to our expectation,
250 ensemble overlap in the OT group was very low, comparable to the $\Delta 24$ h HC group. In
251 contrast, the RT group showed a significantly higher overlap than the $\Delta 24$ h HC group. The
252 spatial search consistency between the two tagging days was high in the OT group, but
253 ensemble reactivation was low. In contrast, spatial search consistency in the RL was low, but
254 the ensemble reactivation was high. These results indicate that the pattern of cFos+ cells in
255 DG contains little information about the spatial position of the animal.

Figure 6. cFos-tagged GCs poorly reflect mouse location. **a**, Experimental design. TetTag mice were injected bilaterally in DG with AAV-TRE-iChloC-mKate. Mice were trained in the WM with the platform in E for 4 consecutive days with on-Dox. On day 5, mice were trained with the same platform position off-Dox. Mice were put back on-Dox and on day 6 they were either trained again with the platform in E (overtrained, OT) or with the platform in W (reversal training, RT). Mice were sacrificed 3h after the last trial for cFos-tagged GCs immunofluorescence assay. **b**, Escape latencies show similar learning in both groups on day 5. The RT group significantly reduced the escape latency during day 6 (*, $p < 0.05$, repeated measures one-way-ANOVA test). **c**, Heatmaps represent the PTs on day 5 and 6 for the OT and RT groups **d**, Raster plot of time spent at the W annulus (pink), E annulus (blue), and outside the annuli (gray) for each mouse of the OT and RT groups during the PT on day 5 and 6. Numbers on the right indicate platform crossings. **e**, Time in each annulus during the PTs. On day 5, mice show similar performance in both groups with a highly significant preference for annulus E. On day 6, the RT group shows no significant preference for any annulus, while the OT group keep their preference for annulus E (*, $p < 0.05$, t test). **f**, Overlap/chance is significantly higher in the RT than the $\Delta 24$ HC group (**, $p = 0.006$, unpaired, two-tailed t-test).

Discussion

We used activity-dependent expression of a light-gated chloride channel to evaluate how cFos+ ensembles in the dentate gyrus contribute to spatial memory. Optogenetic inhibition of ensembles that were active during early training (day 1) improved memory recall in overtrained animals (day 5) and improved memory acquisition of a new platform location (reversal training, day 6). Even in animals very familiar with the WM, cFos+ ensembles in DG strongly segregated over time, indicating that the pattern of cFos+ expression does not represent the animal's location in space. However, when spatial learning took place, either at early training stages or during reversal training, cFos+ ensembles were partially reactivated. The slow dynamics of cFos+ ensembles in DG may reflect its role in time stamping of memories.

How does DG contribute to spatial memory?

Lesion studies and pharmacological experiments first established that the DG is important for the encoding of spatial memories, but provided no information about activity patterns during navigation^{31,32}. More recently, optogenetic tools were employed to investigate DG function by hyperpolarize hilar interneurons³³, adult-born granule cells³⁴ or projections from the entorhinal cortex³⁵ during water maze training. Optogenetic hyperpolarization of GABAergic interneurons removes the lateral inhibition of GCs, leading to a relatively dense activity of GCs and compromised spatial memory recall³³. Direct hyperpolarization of adult-born GCs or block of the inputs from layer II of medial entorhinal cortex by axonal hyperpolarization also impaired spatial memory recall^{34 35}. These still fairly global interventions suggest that both too dense and too weak GC activity degrades behavioral performance. In addition to water maze experiments, active place avoidance has been used

to study the importance of DG for spatial learning³⁶. In conflict trials, animals with inhibited dorsal DG often entered the shock zone and spent less time in the new ‘safe’ quadrant, indicating impaired discrimination. In contrast, we found that optogenetic inhibition during reversal training improved reversal learning (Fig. 3). There are, however, major differences in the experimental design: While Kheirbeck et al. used a global expression strategy (Pomc-cre) to drive eNpHR3.0 in all GCs, we expressed the optogenetic inhibitor only in the small subset of GCs (~10%) that were active during the first WM-encoding episode. Therefore, Kheirbeck et al. shut down all activity in a large part of the DG while our manipulation left the processing intact in > 90% of GCs, resulting in opposite effects on spatial learning.

The effect of optogenetic silencing depends on the behavioral paradigm

Specific inhibition of previously active neurons in DG has already been used in the context of fear conditioning, a model for posttraumatic stress disorder (PTSD). Optogenetic silencing of arc-tagged ensembles in DG prevented the associated behavioral fear response to a particular encoding context^{18,20}. In contrast, when we silenced the original encoding ensemble in the water maze, we observed *improvement* in the recall of the first platform position (Fig. 2g) and better acquisition of a new spatial location (Fig 3 d-f). Importantly, in contextual fear conditioning experiments, arc-expressing GC populations during induction and recall of fear were overlapping (3-6 fold above chance), while in our spatial memory experiments, the overlap was *below* chance levels. Given the low reactivation between cFos+ ensembles, it is perhaps not surprising that optogenetic silencing of cFos+ neurons from day 1 (first WM-encoding episode) did not suppress spatial memory recall on day 6.

Which factors control cFos+ ensemble reactivation in DG?

Here we report near-constant numbers of cFos+ neurons (ensemble size) under all tested conditions, but consistently higher c-Fos expression (anti-shEGFP fluorescence) in water maze groups compared to open field and home cage controls (Extended Data Figure 4). This suggests spatial navigation does not increase the number of active GCs, but leads to higher activity levels within the active subset. When comparing the impact of environment, task, and elapsed time on the ensemble reactivation, it became clear that time is the dominant factor driving cFos ensembles apart (Fig. 7). However, time is not the only extrinsic factor controlling GC reactivation. Comparing ensemble overlap in all experimental groups with a

Figure 7. Summary cFos+ GCs-tagging experiments, ordered by decreasing ensemble overlap. Elapsed time (Δt) is the strongest predictor of ensemble overlap. Within the $\Delta t = 24$ h groups, overlap above chance level occurred in situations where learning took place. Other task-related (novelty, interference, familiarity) or behavioral parameters (spatial search consistency) were not correlated with ensemble overlap.

time interval of 24 h between the two ensembles ($\Delta 24h$), we noted that relatively high reactivation occurred in situations where the learning curve was very steep (ET: 14%, RT: 12%). Low reactivation (below chance) was found in familiar environments (HC: 10%, OT: 9%) but also in entirely novel environments (NE: 8%), generally in situations where a recall of yesterday's events would have had no benefit for the animal (Fig. 7). The strong influence of time over cFos+ ensemble selection supports the hypothesis that DG processes a familiar environment as novel when experienced temporally separated to aid detecting new features of a known environment for pattern separation^{8,37}. Strikingly, in our results, environmental familiarity alone did not produce ensemble overlap above chance in over trained animals (OT group) even when cFos+ maps were generated only one day apart (Fig. 6). One hypothesis is that cFos+ cells on day 5, when the animals were already experts in navigating the maze and did not improve their performance any more (asymptotic performance), did not carry information required for solving the task. During reversal training on day 6, mice partially reactivated the day 5 ensemble. A plausible interpretation is that mice facing an unexpected situation try to recall past experiences to develop a new strategy. The low overlap in the OT group was quite surprising because it has been reported that GCs with strong place preference have highly stable place fields in familiar environments^{7,10,13}. Our results suggest that the pattern of cFos activity in DG does not indicate the position of the animal or the goal, at least not in the form of labeled line code. It could be argued that cFos+ neurons from early training are still active in the second labeling window, but not enough to drive cFos³⁸. However, when we suppressed activity in the ensemble expressing cFos during initial training via optogenetic inhibition (Fig. 2g), animals were perfectly capable of swimming to the correct platform position. At day 5 of WM training, we even saw slightly

enhanced performance compared to non-inhibited trials. This would not be expected if the cFos+ cells from day 1 still encoded specific locations in the water maze on day 3 and 5.

cFos is part of a mechanism that selects neurons for memory storage.

When we evaluated shEGFP expression levels across home cage groups (Fig. 2), we found that double-positive neurons (shEGFP+ / mKate+) in the $\Delta 0$ h group had the highest fluorescence intensity, even higher than the WM groups (Fig. 4f, Extended Data figure 4). The effect was less pronounced in the $\Delta 6$ h group and even less in the $\Delta 24$ h group. In contrast, shEGFP expression levels in non-reactivated neurons (mKate-) were similar in all home cage groups (Fig. 4f). This observation suggests that DG neurons that were active early in the off-dox period (accumulating mKate) also had the highest activity levels towards the end (resulting in very high shEGFP expression), but not 24 hours later. It is very likely that the reported hyperexcitability of cFos-expressing neurons, caused by K⁺ channel internalization³⁹, is responsible for the correlation (at 0 and 6 h) between the fast/transient (shEGFP) and slow/permanent (mKate) cFos reporter. It is less clear how the period of elevated activity is terminated. High expression of another immediate early gene, homer1a, has been shown to trigger cell-wide synaptic downscaling⁴⁰. As it takes several hours for this homeostatic mechanism to take effect, it is a good candidate to explain the slow migration of DG ensemble activity over time. Importantly, this type of cell-autonomous oscillation would provide a clock that reduces synaptic drive specifically for the most active subset of neurons but has little effect on the 90 % of GCs that are cFos-negative. A cell-specific negative feedback loop between immediate-early gene (IEG) activation and excitatory drive could also explain why we saw overlap below chance levels in several experimental groups, which can only be explained if information about the identity of once-active (cFos+) cells is still

present in the system. Time-dependent switching to a new set of active GCs might indeed be crucial to distinguish similar experiences that occurred separated in time^{41,42}. In hippocampus⁴³ and amygdala⁴⁴, IEG-expressing neuronal ensembles fail to encode two different events as different experiences when they occur close in time (<6 h). Spatial or auditory cues were only discriminated when associated with experiences that occurred distant in time. These observations and our data suggest that the temporal shift in ensemble selection occurs even when the spatial context remains the same. Thus, cFos is not only an indicator of intense neuronal activity but also reflects a clock signal^{6,45} that is crucial for LTP, perhaps selecting a specific subset of neurons for memory storage. Our results support the concept that cFos+ neurons in DG encode an episode, but not a spatial position⁴⁶. The disconnect between cFos expression and place cell activity has also been reported in a recent study of CA1 pyramidal cells⁴⁸ and is therefore not a specific property of DG granule cells.

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472

Methods.

Experimental animals

c-Fos::tTA/cFos::shEGFP (TetTag) mice were obtained from the Jackson Laboratory and bred heterozygous crossed with C57BL6/J mice. Mice were group-housed with littermates until 2 weeks before AAV injections, then were single-caged and changed to doxycycline-containing food. Mice had access to food and water *ad libitum* and were kept in an animal facility next to the behavioral rooms on a reversed light-dark cycle (dark 7 am - 7 pm). All behavioral experiments were done at daytime during the dark phase of the cycle. Due to the weight of the implant and patch-cords (optogenetics), only male mice, between 20-60 weeks (>28g by the time of surgery) were included in the OptoWM experiments (Fig. 2-3). Both male and female mice were included in the cFos ensemble overlap experiments (Fig. 1, 4-6). All experiments were conducted in accordance with the German and European Community laws on protection of experimental animals and approved by the local authorities of the City of Hamburg (Behörde für Gesundheit und Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen, Nr. 100/15).

Viral constructs

pAAV-TREtight-iChloC-mKate was cloned from a backbone provided by Olaf Pongs. The iChloC sequence was taken from a CaMKIIa-iChloC-2A-tDimer²⁶ plasmid and linked to mKate for membrane fluorescent localization. iChloC-mKate was then cloned into the pAAV-TREtight promoter backbone. Virus production was done in the UKE Vector Facility packing the construct into an AAV 2/9 serotype.

495 Surgical procedures

496 To avoid hypothermia, a heating mat was placed under the animal throughout the whole
497 surgery and under its cage for 1 h until full recovery. We provided post-surgery analgesia
498 with Meloxicam mixed with softened Dox-food and left in a Petri dish in the cage for 3 days
499 after surgery. Animals had at least 2 weeks for recovery before behavioral experiments.

500 **AAV injection.** TetTag mice were injected under analgesia and anesthesia using a
501 stereotactic drill and injection robot (Neurostar). Mice were fixed to the frame under
502 isoflurane anesthesia (1.5%, mixed in O₂), skin and connective tissue was removed and two
503 craniotomies were performed using an automated drill on the desired coordinates. AAV9-
504 TRE-iChloC-mKate2 (1×10^{13} vg/ml) was delivered bilaterally into the dorsal dentate gyrus
505 using a glass pipette attached to a 5 ml Hamilton syringe. A single injection per site was
506 performed using stereotaxic coordinates (-2.2 AP, $\pm$ 1.37 ML, -1.9 DV) with a volume of 500-
507 800 nl on each side (injection speed: 100 nl/min). After injection, the pipette was retracted
508 200 μ m and left for at least 5 min to avoid retroflux. After the injections, the bone surface
509 was cleaned with NaCl 0.9% solution and skin was stitched.

510 **Optic fiber implant.** Mice in the optogenetic experiments were implanted with a bilateral
511 optic fiber implant (Doric) targeting the DG sulcus (-2.2 AP, $\pm$ 1.37 ML, -1.5 DV) right after
512 AAV injection. Implant was attached using C&B dental cement onto the skull's surface and a
513 protective cap was made out of an Eppendorf tube. The cap was secured then by applying
514 acrylic resin (Pattern Resin LS, GC America) on all the exposed skull's surface in the implant
515 surroundings.

516 Doxycycline treatment

517 Animals were given doxycycline-containing food (Altromin-Dox, 50 mg per kg of body
518 weight, red pellets). To tag the first cFos ensemble, animals were changed to regular
519 doxycycline-free food (Altormin, light-brown pellets) 24 h before exposure to the desired
520 task. To assure that the animals did not consume any Dox-food crumbs that had fallen into
521 their cages, the bedding and cage were changed together with the food. The old nesting
522 material was transferred to the new cage to decrease novelty-induced cFos expression. Dox
523 food was given back exactly 24 h after removal, right before the behavioral task.

524 Behavioral experiments

525 All experiments were strictly timed due to cFos expression dynamics and temporal ensemble
526 shift (Fig 4). For optogenetic experiments, mice were operated in batches of 6, and tested
527 weeks apart to perform the experiments at similar times of the day. For ensemble overlap
528 experiments, mice were injected in batches of 10 and randomized to each of the groups.
529 Mice that did not have adequate viral transfection, the implant location was off target, or
530 performed poorly (floaters or implant intolerance) were not included in the analysis and
531 therefore not every group has the same 'n'. All experiments were recorded on digital video
532 and automated mouse tracking was done with Ethovision XT 11.5.

533 **Water Maze (WM):**

534 Animals were handled by the experimenters (LA, PL) 1 week before the start of the pre-
535 training sessions to reduce stress during behavioral tasks. **Pre-training.** Mice were pre-
536 trained for 2 days before ever visiting the WM arena (see below). Sessions (3 x 60 s trials on
537 two days) were done in a small rectangular water tank in the dark, in the same room where

the WM task was performed. Water level was 1 cm above the 10 cm diameter escape platform. The position of the platform was alternated between the left and right side of the tank between trials, keeping a distance of 5 cm from the walls to avoid thigmotaxis. Once the animals found the platform, a grid was presented until the animals climbed onto it and were returned to their home cages in the waiting area of the behavioral room. **Training.** The WM consisted of a circular tank (1.4 m diameter) with visual asymmetrical landmarks⁴⁸, filled with water mixed with non-toxic white paint to prevent the animals from seeing the platform (submerged by 1 cm). The platform was placed in the center of the east quadrant during regular training and switched to the opposite (west) quadrant for reversal training (max. training length: 90 s). To test spatial reference memory, a probe trial without a platform was performed on each day. Mice underwent six trials every day (4 training trials (TT, 90 s) + 1 probe trial (PT, 60 s) + 1 TT), inter-trial interval (ITI) was 8-10 s. For the TTs, mice were lowered into the tank facing the wall in different, pseudo-randomized positions (avoiding the target quadrant). In the PT, mice were lowered in the center of the tank. An opaque cup-sized chamber attached to a pole was used to transfer the mice from their home cage to the drop position and a plastic grid attached to a pole was used to pick up the mice. Mice were picked up 10 s after they found the platform and were returned to their home cage. Mice that did not find the platform during the length of the TT, were guided to the platform using the grid and were picked up after a 10 s on-platform waiting period. For both pre-training and WM, water temperature was 19°C and a heating lamp was placed on the waiting area to avoid hypothermia.

559 **Home caged and open field control experiments:**

560 To evaluate the temporal effect of cFos ensembles, mice were unperturbed in their home
561 cage (HC) during the off-dox period but no behavior was analysed. Mice were either
562 sacrificed right after 24h from Dox removal (0Δ group) or put back on Dox and sacrificed
563 after 6 or 24 h. Both cFos+ tagging events were designed to have the same amount of trials
564 and training length in all experiments. However, for the open field experiment, mice were
565 placed inside a square arena (50 x 50 cm, 50 lux) for 20 min but were kept in the behavioral
566 room the same time as their WM counterparts.

567 **Optogenetic inhibition in the water maze:**

568 Mice were connected to two ultra-thin (200 μm core diameter, 2 m, Doric) optic fibers using
569 ceramic ferrules (Doric Lenses, Canada) and put back to their home cage for at least 5 min
570 before any behavioral task. In pre-training trials (2 days, 3 trials per day), mice were
571 connected and disconnected before every trial to habituate them to the tether. During
572 training, only one mouse was tethered at a time so trials were not intercalated. Instead, one
573 mouse was trained from beginning to end before the next one. This allowed training of only
574 6 mice per day. To avoid stress-related cFos tagging, mice were never tethered during the
575 off-Dox period. From day 1-5, mice only received light during two PTs in a counterbalanced
576 light ON/OFF fashion (day 3 & 5, Fig. 2). On day 6, only mice in the RT-S receive light during
577 the reversal TTs (rTT), but not during PTs. Mice in the RT-C did not receive light on day 6 (Fig.
578 3). **Training protocol.** Day 1 (Off-Dox): no tether, 4TT + 1 PT + 1TT; Day 2: tether, 3TT + 1PT +
579 1TT; Day 3: 2PTs tethered + 3TTs without tether; Day 4: 4TT + 1PT + 1TT(tethered); Day 5:
580 tethered, 2TT + 2PTs; Day 6: tethered, 4 rTTs + 1 PT). In all trials with tether, the weight of
581 the fibers was compensated with a white helium balloon (0.07 N pull force), attached to a

582 light fiber ~30 cm above the mouse with a transparent fishing line long enough to keep the
583 balloon out of the field of view of the video camera. The I.T.I. was 10-15 min. Mice were
584 brought back to their HC after each trial but were not untethered. The HC was placed close
585 to the WM tank but behind a curtain on a mobile platform to alternate the exit location in a
586 pseudo-random fashion. Blue light (473 nm) was delivered using a laser combiner (LightHUB,
587 Omicron) connected to a commutator (FRJ_1x2i_FC-2FC_0.22 Doric Lenses, Canada) to split
588 the output into two fibers. The commutator was placed close to the ceiling, at the same
589 height as the camera. To ensure inhibition before and after mice were in the tank, light was
590 delivered in 20 ms pulses repeated at 1 Hz during 120 s for TTs and rTTs (90 s). For PTs (60 s),
591 light was delivered for 90 s. Mice were always sacrificed 1.5h after the last trial.

592 Ex vivo brain processing and cFos immunofluorescence (IF) staining

593 Mice from all behavioral groups were sacrificed 3.5 h after the last trial for shEGFP IF
594 staining (1.5 h after for cFos IF staining) by ketamine/xylazine (100/10 mg kg⁻¹)
595 intraperitoneal injection and intracardially perfused with 1x phosphate-buffered saline (PBS,
596 Sigma) followed by 4% paraformaldehyde (PFA, Roth). Brains were extracted and stored in
597 4% PFA for at least 24 h at 4°C. Before sectioning, brains were washed with 1xPBS for 20 min
598 at room temperature (RT). The dorsal hippocampal region (-1.2 to -2.3 AP from bregma) was
599 cut in 40 µm coronal sections using a vibratome (Leica VT100S) and collected in PBS. From
600 each series, six sections were selected from Bregma -1.7 to -2.3 mm and incubated in
601 blocking buffer (1x PBS, 0,3% TritonX, 5% goat serum) for 2 h at RT. Next, sections were
602 placed in the primary antibody carrier solution (1x PBS, 0,3% TritonX, 1% goat serum, 1%
603 BSA) and incubated overnight at 4°C. After 3x washing with 1xPBS for 5 min, the sections
604 were incubated for 2 h at RT in the secondary antibody carrier solution (1x PBS, 0,3%

605 TritonX, 5% goat serum). Sections were washed 3 x 10 min in 1x PBS, stained with 4',6-
606 diamidino-2-phenylindole (DAPI, 1:1000) for 5 min and mounted on coverslips using Immu-
607 Mount (Shandon). Primary antibodies: Chicken Anti GFP polyclonal antibody, Invitrogen
608 (A10262, Lot 1972783); Rabbit Anti-tRFP (mKate2) antibody, Evrogen (AB233, Lot
609 23301040466), Rabbit anti cFos, Synaptic Systems (226003, Lot). Secondary antibodies:
610 Alexa Fluor 488 conjugated secondary antibody (goat anti-chicken, 1:1000, Life technologies;
611 A11039; Alexa Fluor 568 conjugated secondary antibody (goat anti-rabbit, 1:1000, Life
612 Technologies, A11011).

613 Confocal imaging

614 Before imaging, mounted slides were labeled with a code to blind the analyst. The stained
615 slices were imaged with a confocal microscope (Olympus Fluoview FV 1000) using a 20x oil
616 immersion objective (UPLSAPO 20X NA: 0.85). From each of the 6 sections per brain, a stack
617 of 6 images (step size 2.5 μ m) from both dentate gyri was taken. 3 channel images were
618 obtained at a 1024x1024 resolution. Excitation/emission filters were selected using the dye
619 selection function of the Olympus software (Alexa 405 (DAPI), Alexa 488 (shEGFP) and Alexa
620 568 (mKate)). The image acquisition settings were optimized once and kept constant for all
621 images within an experimental data set. Images were not deconvolved, gamma-adjusted or
622 filtered. Example images presented in figures, the look-up-table was linearly adjusted,
623 filtered with a 2-pixel median filter and cropped to show relevant regions.

624 Ensemble size calculation and overlap analysis

625 Confocal image stacks (green: shEGFP; pseudo-colored magenta: mKate; blue: DAPI) were
626 analyzed using Imaris (Bitplane). The DAPI channel was used to create a surface of the upper
627 and lower blade of DG (granule cell layer, GCL). The volume of the GCL was used to estimate

the total GC number based on published cell densities⁴⁹ and to mask the green and the pseudo-colored magenta channel to restrict analysis to the GCL. Automatic spot detection was used to identify shEGFP+ cells in the green channel. The quality filter (round nuclei with a diameter of 8 µm) was once adjusted by the analyst to include weakly expressing cells and then applied to every image stack of the same experiment. False positive spots (e.g. staining artifacts) were manually removed. It was not possible to detect mKate-expressing GCs automatically, as only the plasma membrane was labeled. To count mKate-positive cells, spots were placed manually (spot size 10.6 µm) using the pseudo-colored magenta channel only. Double-positive cells (distance between shEGFP+ and mKate+ spots < 5 µm) were identified using a Matlab script. They were then manually inspected to check for artifacts and, if necessary, corrected. From the Imaris analysis, we calculated the following values using Excel spreadsheets:

$$\text{Number of granule cells} = \frac{\text{DAPI surface volume}}{\text{reported GC density}}$$

$$\text{fraction of shEGFP tagged cells (\%)} = \frac{\text{number of shEGFP + cells}}{\text{number of GCs}}$$

$$\text{fraction of mKate tagged cells (\%)} = \frac{\text{number of mKate2 + cells}}{\text{number of GCs}}$$

$$\text{fraction double tagged cells (\%)} = \frac{\text{number of double positive cells}}{\text{number of GCs}}$$

$$\text{expected reactivation} = \text{fraction of shEGFP cells} * \text{fraction of mKate cells}$$

$$\text{Reactivation above chance} = \frac{\text{number of double positive cells}}{\text{expected reactivation}}$$

646

647 Preparation of hippocampal slice cultures

648 Hippocampal slice cultures from TetTag mice or Wistar rats were prepared at postnatal day 8
649 or 4–7 respectively as described⁵⁰. Briefly, animals were anesthetized with 80% CO₂ 20% O₂
650 and decapitated. Hippocampi were dissected in cold slice culture dissection medium
651 containing (in mM): 248 sucrose, 26 NaHCO₃, 10 glucose, 4 KCl, 5 MgCl₂, 1 CaCl₂, 2 kynurenic
652 acid, 0.001% phenol red (310–320 mOsm kg⁻¹, saturated with 95% O₂, 5% CO₂, pH 7.4). Tissue
653 was cut into 400 μM thick sections on a tissue chopper and cultured at the medium/air
654 interface on membranes (Millipore PICMORG50) at 37 °C in 5% CO₂. No antibiotics were added
655 to the slice culture medium which was partially exchanged (60–70%) twice per week and
656 contained (for 500 ml): 394 ml Minimal Essential Medium (Sigma M7278), 100 ml heat
657 inactivated donor horse serum (H1138 Sigma), 1 mM L-glutamine (Gibco 25030-024), 0.01 mg
658 ml⁻¹ insulin (Sigma I6634), 1.45 ml 5 M NaCl (S5150 Sigma)), 2mM MgSO₄ (Fluka 63126), 1.44
659 mM CaCl₂ (Fluka 21114), 0.00125% ascorbic acid (Fluka 11140), 13 mM D-glucose (Fluka
660 49152).

661 Calibration of iChloC in dentate granule cells of organotypic cultures

662 Organotypic rat slice cultures were infected with AAV2/9 pAAV-CaMKII-ChR (E83Q, E90R,
663 E101S, D156N, T159C)-2A-tdimer2 (iChloC) 81-2 (Virus titer: 3.75x10¹²) after 28 days in vitro
664 (DIV). At DIV 36, patch clamp recordings of dentate granule cells were performed using a BX-
665 51WI microscope (Olympus), a Multiclamp 700B amplifier (Molecular Devices), a motorized
666 micromanipulator MP-285 (Sutter Instruments) and Wavesurfer software programmed with
667 Matlab (The MathWorks). Slices were placed in HEPES-buffered solution (21-23 °C) consisted
668 of (in mM): 145 NaCl, 10 Na-HEPES, 12.5 D-glucose, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄

(pH 7.4). Patch pipettes with a tip resistance of 3–5 MΩ were filled with intracellular solution (in mM): 135 K-gluconate, 4 MgCl₂, 4 Na₂-ATP, 0.4 Na-GTP, 10 Na₂-phosphocreatine, 3 sodium-L-ascorbate and 10 HEPES (pH 7.2). A LED light engine (SpectraX, Lumencor) was used for delivery of light pulses (476 nm) through a 60x (Olympus) objective. Light intensity was measured with a calibrated power meter (LaserCheck, Coherent) and divided by the illuminated field of view (0.169 mm²) to achieve 10 mW/mm². The AP threshold was determined by injecting current steps from 0 - 150 pA. Patched-clamped cells were stimulated every 5 s with a 20 ms 150 pA square pulse for 60 seconds. 20 ms before stimulation 4, 7, or 10 light pulses (20 ms, 476 nm, 10 mW/mm²) were applied. Series resistance was between 10 and 40 MΩ.

Statistical analysis

The definition and the exact value for n is given in the text or in figure legends. Replication of experiments is reported in figure legends. Statistical analysis was performed using GraphPad Prism (v8). Performed statistical tests are described in each figure legend.

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692 Author contributions

693 PLM, AKE, FM and TGO conceived and designed experiments. PLM, AF, and LA performed
694 behavioral experiments. PLM, JA, LL, and AF performed electrophysiological experiments.
695 PLM, AF, JA, LL, and FM analyzed data. MK, AKE and TGO provided infrastructure and
696 equipment. PLM, FM and TGO wrote the paper. All authors edited and approved the
697 manuscript.

698 Competing interests

699 The authors declare that they have no competing interests.

Figures

Figure 1

General experimental design for tagging two c-Fos+ neuronal ensembles suitable for WM training. a, TetTag mice containing two cFos-driven transgenes: 1) tetracycline trans-activator (tTA) and 2) nuclear-localized, short-life enhanced green fluorescent protein (shEGFP). To tag (and manipulate) cFos+ cells,

TetTag mice are injected bilaterally in DG with an AAV containing the improved version of a chloride-conducting channel rhodopsin (iChloC) under the control of a tTA response element (TRE). Doxycycline (black triangle) prevents tTA from activating TRE-iChloC-mKate expression. When Dox is removed, active GCs express TRE-iChloC-mKate. shEGFP expression is independent of Dox. b, Experiment timeline. TetTag mice were injected with AAVs in DG on-Dox. Two weeks later, Dox was removed for 24 h (Dox-off). Some mice were in their home cage (HC) and sacrificed 24 h after Dox removal (0d, n = 3) or 48 h after Dox removal (1d, n = 3). A different cohort of mice experienced water maze (WM) training off-Dox. After training, mice were put back on Dox and were either sacrificed 48h after Dox removal (1d, n = 4) or 6 days after (6d, n = 7). c, Confocal images showing DG. All tagged-GCs were counted within the limits of the GC layer (DAPI). iChloC-mKate expresses on GC membrane (pseudocoloured in magenta) while shEGFP (green) is only in the nuclei. Overlapping cells show magenta membrane and green nuclei. d, Confocal image set showing both GCs ensembles for all conditions. Dox prevents iChloC-mKate expression. f, Ensemble size (mKate+/DAPI and shEGFP+/DAPI). mKate+ ensemble size is significantly lower in the 0d than 1d (*, $p=0.03$, Brown-Forsythe ANOVA, Dunnet multiple comparisons 0d vs. 1d). mKate+ ensemble size was similar regardless of experience (HC or WM) 1d after Dox removal. mKate ensemble size was not decreased 6 days after tagging. shEGFP ensemble size was similar regardless of experience (HC or WM) or day of sacrifice.

Figure 2

Optogenetic silencing of day 1 cFos-tagged DG ensembles improves spatial memory recall in over trained (day 5) mice but not at early training (day 3) phases of the WM. a, TetTag mice were injected with AAV-TRE-iChloC-mKate2 and a bilateral fiber-optic cannula was implanted above DG. For optogenetic inhibition, 20ms (1Hz), 473nm light pulses were used. b, Setup for optogenetic stimulation in the water maze, using calibrated pull-up by helium balloon. c, Experimental design. Mice (n = 16) were trained in the

WM with a platform in quadrant E, on day 1, off-Dox and put back on-Dox afterward (tagging day). On day 2, mice were trained again with tether, but no optogenetic manipulation was done (early training). Before day 3 training, mice were tethered and were tested twice (probe trial light ON/OFF) for reference spatial memory. Mice were trained untethered 4. On day 5, mice had 2 probe trials with and without light. d, Schematic representation virtual divisions of the WM. e, Escape latency at different stages of training. Mice reach asymptotic performance on day 5. f, Counterbalanced (light OFF/ON) probe trials on day 3. Mice show a significant preference for annulus E over W (****, $p < 0.0001$, effect of annulus position by mixed 2-way-ANOVA) but no interaction between light and time in the annulus ($p = 0.47$, matched, 2-way-ANOVA). g, Counterbalanced (light OFF/ON) probe trials on day 5. Mice show a significant preference for annulus E over W (****, $p < 0.0001$, effect of annulus position by mixed 2-way-ANOVA) and light significantly increases the spent time in annulus E (* $p = 0.04$, Bonferroni test after mixed 2-way ANOVA: annulus E, light OFF vs. ON). h, Swim speed and distance swum are not affected by light on day 5. i, Hippocampal slice cultures were used to test the effect of blue light on action potential formation in GCs expressing iChloC.

Figure 3

Optogenetic silencing of day 1 cFos-tagged DG ensembles improves reversal training acquisition and recall. **a**, Experimental design. The same cohort of mice from Fig. 2 was separated into two groups with similar performances during the last light OFF PT on day 5. Both groups were tethered and underwent four training trials with the platform in annulus W (reversal training, RT). RT-silenced mice received 20 ms, 473 nm light pulses during the whole length of each learning trial. 1.5h after RT, mice were sacrificed for

cFos ensemble immunofluorescence essay. b, Escape latency of each group (only trial 1 and 4 shown). c, Schematic representation of WM tank virtual divisions. d, Shift in search behavior over RT trials on day 6. Heatmaps showing average mouse position for the RT-C (top row) and RT-S (bottom row) groups. Mean minimal distance to the new platform position (W) over the four RT trials. The RT-S group, but not the RT-C group, swim closer to the new platform position during trial four compared to trial one (***, $p=0.0004$ trial; Tukey's test after mixed 2way ANOVA). e, PT 60s, no platform, no light, tethered. Both groups showed similar spatial memory retrieval but mice in the RT-S group showed no longer preference for annulus E over W while RT-C does (*, $p=0.02$ trial; Tukey's test after mixed 2-way ANOVA). f, Raster plot of behavior per individual mice during the PT (60 s). Time spent in annuli (W shown in pink, E shown in blue) gray indicates time spent outside annuli. Numbers on the right indicate platform crossings. PT divided in 30 s time bins shows mice in the RT-S group search in both annuli while mice in the RT-C show a significant preference for annulus E in the first 30s of the PT (** $p=0.009$, test 0-30s vs. 30-60s, Bonferroni's multiple comparisons after mixed 2-way ANOVA). g, cFos-tagged cells from total of DAPI cells from DG GC layer. Both groups show similar ensemble sizes for day 1 (mKate+) and day 6 (shEGFP+). h, overlap/chance was significantly lower in the RT-S group (*, $p=0.03$, unpaired t-test). i, Venn diagram shows ensemble overlap is minimal in both groups.

Figure 4

In the familiar home cage (HC), cFos+ GC ensembles strongly segregate over time. **a**, Experimental design: TetTag mice were injected with AAV-TRE-iChloC-mKate. Dox was removed (off) for 24h and mice were sacrificed immediately after (0h group, n = 3). Dox was given back (on) and mice were either sacrificed 6 (6h group, n = 3) or 24h after (24h group, n = 4). **b**, Representative confocal images of cFos-tagged GCs. cFos-tagged cells during the off-Dox period express mKate (pseudo-colored in magenta). Transient tagged cFos GCs from hours prior to sacrifice express shEGFP (green). **c**, Venn diagram of tagged cells show ensemble overlap. **d**, Overlap/chance is similar in the 0h and 6h groups (ns, p = 0.11, unpaired t-test). Both the 0h and 6h groups have significantly higher overlap/chance than the 24h group (****, p < 0.0001, unpaired t-test). **e**, Overlap/chance follows a sigmoidal function over time. **f**, Double-labeled GCs (mKate+/shEGFP+) expressed shEGFP significantly stronger immediately after the

bedding change (0h) compared to 6 h later (*, $p = 0.01$), or 24 h later (****, $p = 0.01$). In GCs expressing only shEGFP, not mKate, expression levels were identical at all time points (two way-ANOVA, Tukey).

Figure 5

cFos+ GC ensembles get partially reactivated during early training phases of WM but segregate when mice visit an unfamiliar environment. **a**, Experimental design. TetTag mice were injected with AAV-TRE-iChloC-mKate in DG and trained while Dox (-) in the WM (platform in E). Mice were then put back on Dox (+) and 24h later, they were either trained again in the WM (early training, ET) or visited an open field arena in a different room for 20 mins (novel environment, NE). Mice were sacrificed 3h after the last trial for cFos-tagged GCs immunofluorescence assay. **b**, Escape latencies from the first and last trial of day 1, show similar learning in both groups. Mice in the ET group significantly reduced their escape latency from day 1 to day 2 (*, $p < 0.05$, repeated measures one-way-ANOVA test). **c**, Reference memory PT, no platform. Heatmaps from each day per group. Representative OF track of one mouse from the NE group on day 2. **d**, Quantification of time in each annuli during PT. On day 1, mice show similar spatial reference memory

performance in both groups but no significant preference for annulus E. On day 2, mice from the ET group show a significant preference for annulus E(*, $p < 0.05$, RM-two way-ANOVA). e, epretentative confocal images of DG GC layer. ET, top; NE bottom. Arrows indicate GCs with cFos on both days. f, Ensembles size. Both groups show similar ensembles size for both days. g, Overlap/chance is significantly higher in the ET group than the NE or HC 24h group from Fig. 4. (*, $p < 0.05$, t test)

Figure 6

cFos-tagged GCs poorly reflect mouse location. a, Experimental design. TetTag mice were injected bilaterally in DG with AAV TRE-iChloC-mKate. Mice were trained in the WM with the platform in E for 4 consecutive days with on-Dox. On day 5, mice were trained with the same platform position off-Dox. Mice were put back on-Dox and on day 6 they were either trained again with the platform in E (overtrained, OT) or with the platform in W (reversal training, RT). Mice were sacrificed 3h after the last trial for cFos-tagged GCs immunofluorescence essay. b, Escape latencies show similar learning in both groups on day 5. The RT group significantly reduced the escape latency during day 6 (*, $p < 0.05$, repeated measures one-way-ANOVA test. c, Heatmaps represent the PTs on day 5 and 6 for the OT and RT groups d, Raster plot of time spent at the W annulus (pink), E annulus (blue), and outside the annuli (gray) for each mouse of the OT and RT groups during the PT on day 5 and 6. Numbers on the right indicate platform crossings. e, Time in each annulus during the PTs. On day 5, mice show similar performance in both groups with a highly significant preference for annulus E. On day 6, the RT group shows no significant preference for any annulus, while the OT group keep their preference for annulus E (*, $p < 0.05$, t test). f, Overlap/chance is significantly higher in the RT than the 24 HC group (**, $p = 0.006$, unpaired, two-tailed t-test).

Supplementary Files

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