Astrocytes contribute to remote memory formation by modulating hippocampal-cortical communication during learning

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Remote memories depend on coordinated activity in the hippocampus and frontal cortices, but the timeline of these interactions is debated. Astrocytes sense and modify neuronal activity, but their role in remote memory is scarcely explored. We expressed the Gq-coupled designer receptor hM4Di in CA1 astrocytes and discovered that astrocytic manipulation during learning specifically impaired remote, but not recent, memory recall and decreased activity in the anterior cingulate cortex (ACC) during retrieval. We revealed massive recruitment of ACC-projecting CA1 neurons during memory acquisition, which was accompanied by the activation of ACC neurons. Astrocytic Gq activation disrupted CA3 to CA1 communication in vivo and reduced the downstream response in the ACC. In behaving mice, it induced a projection-specific inhibition of CA1-to-ACC neurons during learning, which consequently prevented ACC recruitment. Finally, direct inhibition of CA1-to-ACC-projecting neurons spared recent and impaired remote memory. Our findings suggest that remote memory acquisition involves projection-specific functions of astrocytes in regulating CA1-to-ACC neuronal communication.

Remote memories, weeks to decades long, continuously guide our behavior and are critically important to any organism, as the longevity of a memory is tightly connected to its significance. The ongoing interaction between the hippocampus and frontal cortical regions has been repeatedly shown to transform during the transition from recent (days long) to remote memory1–3. However, the exact time at which each region is recruited, the duration for which it remains relevant to memory function and the interactions between these regions are still debated.

Astrocytes are no longer considered to merely provide homeostatic support to neurons and to encapsulate synapses, as pioneering research has shown that they can sense and modify synaptic activity as an integral part of the ‘tripartite synapse’4. Interestingly, astrocytes exhibit extraordinary specificity in their effects on neuronal circuits5 at several levels. First, astrocytes differentially affect neurons based on their genetic identity. For example, astrocytes in the striatum selectively respond to, and modulate, the input onto two populations of medium spiny neurons expressing either D1 or D2 dopamine receptors6. Similarly, astrocytes differentially modulate the effect of specific inhibitory cell types but not others in the same brain region7–10 and selectively affect different inputs to the hippocampus11. Second, astrocytes exert neurotransmitter-specific effects on neuronal circuits. For instance, astrocytic activation in the central amygdala specifically depresses excitatory inputs and enhances inhibitory inputs12. Finally, astrocytes exhibit task-specific effects in vivo; that is, astrocytic stimulation selectively increases neuronal activity when coupled with memory acquisition, but not in the absence of learning13. An intriguing open question is whether astrocytes can also differentially affect neurons based on their distant projection target.

The integration of novel chemogenetic and optogenetic tools in astrocyte research allows real-time reversible manipulation of these cells at the population level combined with electrophysiological and behavioral measurements. Such tools were used in brain slices to activate intracellular pathways in astrocytes and showed their ability to selectively modulate the activity of neighboring neurons in the amygdala and striatum12–14 and to induce de novo long-term potentiation in the hippocampus15–17. Importantly, the reversibility of chemogenetic and optogenetic tools enables careful dissection of the effect of astrocytes during different memory stages in behaving animals16–17. The recruitment of intracellular signaling pathways in astrocytes using such tools is starting to shed light on their complex involvement in memory processes. For example, Gq activation in the CA1 during acquisition (but not during recall) results in enhanced recent memory18, whereas Gq activation results in recent memory impairment18. These findings point to the importance of astrocytes in memory processes, specifically at the time of learning.

To explore the role of astrocytes in memory acquisition, we used the designer receptor hM4Di to activate the Gq pathway in these cells. We show that this astrocytic modulation in the CA1 during learning results in a specific impairment in remote (but not recent) memory recall, which is accompanied by decreased activity in the ACC at the time of retrieval. In vivo, Gq activation in astrocytes disrupts synaptic transmission from the CA3 to the CA1 and reduces downstream recruitment of the ACC. Finally, we reveal a dramatic recruitment of CA1 neurons projecting to the ACC during memory acquisition and a projection-specific inhibition of this population by Gq pathway activation in CA1 astrocytes. Indeed, when we directly inhibited only CA1-to-ACC projecting neurons, recent retrieval remains intact, whereas remote memory is impaired.

Results

Gq pathway activation in CA1 astrocytes specifically impairs the acquisition of remote memory. To specifically modulate
the activity of the Gi pathway in CA1 astrocytes, we employed an adeno-associated virus vector (AAV8) encoding the designer receptor exclusively activated by designer drugs (DREADD) hM4Di fused to mCherry under the control of the astrocytic GFAP promoter. Stereotactic delivery of this AAV8-GFAP::hM4Di–mCherry vector resulted in CA1-specific expression that was restricted to astrocytic outer membranes (Fig. 1a,b), with high penetrance (>85% of GFAP cells expressed hM4Di), and the promoter provided almost complete specificity (>95% hM4Di-positive cells were also GFAP-positive) (Extended Data Fig. 1a,b). Co-staining with the neuronal marker NeuN showed <1% overlap with hM4Di expression (Extended Data Fig. 1c,d).

Recent work has shown that hM4Di activation in astrocytes mimics the response of these cells to GABAergic stimuli14,19 and induces elevated expression of the immediate-early gene cFos in vivo14,15,19. To verify this effect in our hands, mice were injected with clozapine-N-oxide (CNO; 10 mg per kg, intraperitoneally (i.p.)) and brains collected 90-min later and stained for c-Fos. As expected, CNO dramatically increased c-Fos levels in astrocytes of hM4Di-expressing mice compared with saline-injected controls (Fig. 1c). Consistent with the recruitment of the Gi pathway14,19, it seems to be an unreliable indicator of the nature of astrocytic activity as it only indicates the occurrence of a significant modulation. Thus, to better characterize the effect of Gi pathway activation in astrocytes at a time frame more relevant to behavioral experiments (executed tens of minutes after CNO administration), we performed prolonged two-photon imaging in brain slices using Ca2+ levels as a proxy for astrocytic activity. CA1 astrocytes expressing both hM4Di and GCaMP6f were imaged before and after application of artificial cerebrospinal fluid (ACSF) or CNO (10µM) (Fig. 1d,e; Extended Data Fig. 1e–h). CNO application triggered a moderate decrease in baseline intracellular Ca2+ levels in hM4Di-expressing astrocytes (t(10) = 1.8, P = 0.033) (Fig. 1f) and reduced the total size of Ca2+ events in these cells (t(10) = 3.5, P = 0.0005) (Fig. 1g) compared with astrocytes treated with ACSF.

We have shown in the past that CNO alone, without expression of designer receptors, has no effect on calcium activity in astrocytes in the same time frame14. Thus, we find that the reported initial increase in calcium activity in astrocytes following Gi pathway activation14,19, which is sufficient to induce c-Fos expression in vivo (Fig. 1c), is accompanied later by a decrease in calcium dynamics as opposed to GABA-activated astrocyte activity, which results in both acute and minutes-long increases in calcium activity11.

Previous elegant research demonstrated the necessity of normal astrocytic metabolic support to memory and showed that chronic genetic manipulations in astrocytes affect memory acquisition and maintenance31. The contribution of astrocytes to remote memory acquisition, however, was never investigated. To address this topic, we took advantage of the temporal flexibility offered by chemogenetic tools, which allows not only cell-type-specific but also memory-stage-specific (for example, during acquisition or recall) reversible modulation of astrocytes. Indeed, we have recently used such techniques to show that activation of the Gi pathway in astrocytes enhances recent memory acquisition, but has no effect at the time of memory recall11.

To test the effect of astrocytic Gi pathway modulation on cognitive performance, we first injected mice bilaterally with AAV8-GFAP::hM4Di–mCherry into the dorsal CA1. Three weeks later, CNO (10 mg per kg, i.p.) was administered 30 min before fear conditioning (FC) training, which paired a foot shock with a novel context and an auditory cue. CNO application in GFAP::hM4Di mice had no effect on context exploration (Extended Data Fig. 2a) or on baseline freezing before training (Fig. 1b, left) before shock administration. One day later, when CNO was no longer present, mice were placed back in the conditioning context and freezing was quantified. We found no difference in recent memory retrieval between GFAP::hM4Di mice treated with CNO or saline during FC acquisition (Fig. 1b, right). Remarkably, when the same mice were tested in the same context 20 days later, those treated with CNO during conditioning showed a dramatic impairment in memory retrieval (t(11) = 2.2, P = 0.028) (Fig. 1f, left). This deficiency was still clearly observed 45 days after that, when mice were re-tested in the same context for a third time (t(11) = 3.5, P = 0.0025) (Fig. 1f, right). The effect of CA1 astrocytic manipulation was unique to the hippocampal-dependent contextual memory task, as no effect was observed when the same mice were tested for auditory-cued memory in a novel context. That is, both groups demonstrated similar freezing in response to the tone 1 day after training (t(11) = 94.2, time main effect P = 9,97 × 10−10) and 20 days later (t(11) = 13.4, time main effect P = 0.004) (Extended Data Fig. 2b,c).

To verify that the observed effects are not the result of minor off-target hM4Di expression in neurons, we then tested the effects of inhibition of CA1 neurons on recent and remote memory recall. We injected mice with an AAV5-CaMKII::hM4Di–mCherry vector to induce hM4Di expression in ~20% of CA1 glutamatergic neurons (Extended Data Fig. 2d). To test the effect of direct neuronal
inhibition on recent and remote memory acquisition, we injected CaMKII:hM4Di mice with CNO (10 mg per kg, i.p.) 30 min before FC acquisition. Gi pathway activation in neurons had no effect on the exploration of the conditioning cage before tone and shock administration (Extended Data Fig. 2e) or on baseline freezing levels (Fig. 1j, left). Mice were then fear-conditioned and tested on the next day. As expected, neuronal inhibition during training resulted in impaired contextual freezing 1 day later ($t_{17} = 3, P = 0.004$) (Fig. 1j, middle). When the same mice were tested in the same context 20 days later, the memory impairment was still apparent ($t_{17} = 1.8, P = 0.046$) (Fig. 1j, right). No significant effect on auditory-cued memory in a novel context was observed at either the recent or the...
remote time points, as both groups demonstrated similar freezing in response to the tone (time main effect $F_{(1,12)} = 155.4, P = 5.59 \times 10^{-10}$ and $F_{(1,12)} = 34.7, P = 1.77 \times 10^{-3}$, respectively) (Extended Data Fig. 2f,g). Thus, neuronal inhibition during acquisition impairs both recent and remote memory.

Previous reports22–24 have shown effects specific to remote, but not recent, memory in response to neuronal manipulations at the time of recall. Thus, we next tested the necessity of intact astrocytic function during the retrieval of recent and remote memory. CNO administration during recent and remote recall tests of contextual or auditory-cued memory had no effect on freezing levels compared with saline-injected controls (Extended Data Fig. 2h–j). This finding is similar to our previously reported lack of effect of G satisfying pathway manipulation during memory recall25. Thus, normal astrocytic activity is not required during either recent or remote recall, but only during memory acquisition.

To further validate the unexpected effect of astrocytic Gpathway activation during acquisition on remote memory in a less stressful task, we employed the non-associative place recognition (NAPR) paradigm. In this task, mice first explore a novel open field and are then expected to display decreased exploration of this now familiar environment after re-exposure to the same arena. Indeed, GFAP::hM4Di mice injected with either saline or CNO during NAPR acquisition showed a marked decrease in exploration following a second exposure to the square environment to which they were exposed 1 day earlier ($F_{(1,12)} = 45.7, no interaction, time main effect $P = 2.01 \times 10^{-7}$) (Fig. 1k). In a new cohort of GFAP::hM4Di mice, exploration after the second exposure to a round environment that they had originally explored 4 weeks earlier was markedly reduced in mice injected with saline during NAPR acquisition. However, exploration levels in CNO-treated GFAP::hM4Di mice did not decrease (Fig. 1l, left), which suggests that they did not recall their remote experience in this context. These findings were reflected in a significant treatment by time interaction ($F_{(1,11)} = 15.98, P < 0.002$) and a post-hoc analysis showed a significant difference between the first and second visit only for the saline-treated group ($P = 0.001$). A significant effect was also found for the decrease in exploration between saline-treated mice and CNO-treated mice ($t_{(10)} = -2.8, P = 0.0085$) (Fig. 1l, right). To confirm that these mice are still capable of performing NAPR normally when astrocytic activity is intact and to verify the absence of nonspecific long-term effects, we repeated the experiment in a novel trapezoid environment with no CNO administration in the same cohort. The results demonstrated comparable performance between groups ($F_{(1,11)} = 14.89, time main effect $P = 0.003$, no interaction) (Extended Data Fig. 2k).

To verify that our results did not stem from the CNO application itself, control mice injected with an AAV8-GFAP::eGFP vector were trained in the same behavioral paradigms. CNO administration (10 mg per kg, i.p.) in these mice had no effect on baseline freezing, on recent or remote contextual memory or on performance in the remote NAPR task ($F_{(1,11)} = 58.7, time main effect $P = 9.86 \times 10^{-4}$, no interaction) (Extended Data Fig. 3a–d).

Our results show that G activation in CA1 astrocytes during the acquisition of spatial memory selectively impairs its remote, but not recent, recall, whereas direct neuronal inhibition during acquisition impairs both recent and remote memory. These findings raise two novel hypotheses. First, that the foundation for recent memory is established during acquisition in a parallel process separate to recent memory, and can therefore be independently manipulated. Second, that astrocytes are able to specifically modulate the acquisition of remote memory, with precision not granted by general neuronal inhibition. Both hypotheses were tested below.

Astrocytic G pathway activation during memory acquisition reduces the recruitment of brain regions involved in remote memory during retrieval. The transition from recent to remote memory is accompanied by brain-wide reorganization, including the recruitment of frontal cortical regions like the ACC, as indicated by an increased expression of c-Fos23,25. To gain insight into changes in neuronal activity accompanying the recent and remote retrieval of memories acquired under astrocytic modulation, GFAP::hM4Di mice were injected with saline or CNO before FC acquisition. Brains were then collected 90-min after recent or remote recall, stained for C-Fos and quantified in neurons at the CA1 and the ACC (Fig. 2a), two areas that are repeatedly implicated in remote memory. As before, CNO administration to GFAP::hM4Di mice during acquisition had no effect on recent contextual memory (Fig. 2b), and no changes in c-Fos expression following recent recall in either CA1 or ACC were observed (Fig. 2c–e). Another cohort of GFAP::hM4Di mice was injected with CNO before acquisition, tested for recent memory 24 h later and then for remote recall 21 days after that. Importantly, we replicated our initial finding that astrocytic modulation during acquisition specifically impaired remote, but not recent, contextual memory ($t_{(6)} = 2.6, P = 0.014$) (Fig. 2f). Impaired remote memory was accompanied by reduced C-Fos expression in both the CA1 ($t_{(6)} = 2.6, P = 0.0175$) and the ACC ($t_{(6)} = 2.61, P = 0.0175$) regions (Fig. 2g–i). We also performed the same c-Fos quantification in brains collected after the last recall test from the first behavioral experiment (Fig. 1l) of mice that were injected with CNO > 60 days earlier. In this experiment, impaired remote recall in GFAP::hM4Di mice treated with CNO during conditioning was also accompanied by reduced c-Fos expression in the CA1 and the ACC compared with saline-treated mice ($t_{(10)} = 2.01, P = 0.029$ and $t_{(10)} = 1.97, P = 0.04$, respectively) (Extended Data Fig. 4b).

In the same mice we also quantified retrieval-induced c-Fos expression in several additional brain regions known to be involved in memory, such as the dentate gyrus (DG) of the hippocampus, the retrosplenial cortex (RSC) and the basolateral amygdala (BLA). No changes in c-Fos expression in the DG or RSC were observed. BLA c-Fos expression was reduced in GFAP::hM4Di mice treated with CNO ($t_{(6)} = 3, P = 0.011$) (Extended Data Fig. 4a,c). Finally, to exclude any nonspecific effects of CNO itself, we repeated the same experiments in control GFAP::eGFP mice. As before, CNO application alone did not induce a difference in either recent or remote fear memory, and we did not find alterations in c-Fos expression (Extended Data Fig. 4d–k).

Again, we showed that astrocytic G pathway activation during fear memory acquisition selectively impairs remote recall, but spares recent retrieval. Moreover, 3 weeks after manipulation, this memory deficiency is accompanied by reduced activity not only in the CA1, where astrocytes were modulated, but also in the ACC. This temporal association, however, does not necessarily indicate causality, and two possible explanations can be offered: (1) that astrocytic G activation induces a long-term process whose consequences are only observed weeks later or (2) that it acutely impairs the acquisition of remote (but not recent) memory. We tested both options below.

Modulation of CA1 astrocytes has no effect on hippocampal neurogenesis. Our findings of intact recent memory followed by impaired remote memory and reduced hippocampal activity could suggest that astrocytic modulation during acquisition initiates a long-term process that takes weeks to convey its effect. One example of such a process could be hippocampal neurogenesis occurring between recent and remote recall, which has been repeatedly shown to reduce remote memory4. Thus, we sought to examine whether astrocytic manipulation induces changes in neurogenesis. To tag newborn cells, we administered 5-bromodeoxyuridine (BrDU; 100 mg per kg, i.p.) together with the CNO or saline injection to GFAP::hM4Di mice 30 min before acquisition, and another dose 2 h after training. Brains from mice tested for recent retrieval were
stained for BrdU, thereby tagging the cells added to the DG since the previous day. No changes in proliferation or in the number of cells expressing doublecortine (DCx), a marker of young neurons 3 days to 3 weeks old, were observed (Fig. 2j–l). Similarly, in brains collected after remote recall, no changes in the survival of cells formed on the day of acquisition 3 weeks previously or their differentiation fate (as determined by co-staining with the neuronal marker NeuN) were observed. Additionally, no change in the
the ACC are temporally associated (that is, when remote recall is that remote memory performance and c-Fos levels in the CA1 and of the ACC during memory acquisition.

Our findings showed 1234 Gi pathway activation in CA1 astrocytes prevents the recruitment to the selective impairment of remote memory was subsequently investigated.

(Extended Data Fig. 4l–q). Control mice had no effect on neurogenesis 24-h or 21-days later DCx, was observed (Fig. 2m–o). CNO application in GFAP::eGFP number of young neurons born during these 3 weeks, marked by DCx, was observed (Fig. 2m–o). CNO application in GFAP::eGFP control mice had no effect on neurogenesis 24-h or 21-days later (Extended Data Fig. 4l–q).

To conclude, astrocytic manipulation in the CA1 had no effect on hippocampal neurogenesis; therefore, an alternative mechanism to the selective impairment of remote memory was subsequently investigated.

G_i pathway activation in CA1 astrocytes prevents the recruitment of the ACC during memory acquisition. Our findings showed that remote memory performance and c-Fos levels in the CA1 and the ACC are temporally associated (that is, when remote recall is low, so are c-Fos levels at the time of recall), but it is challenging to conclude which phenomenon underlies the other. Furthermore, the temporal distance between the appearance of these phenotypes and the astrocytic manipulation 3 weeks earlier makes it hard to determine exactly when they were induced. We therefore tested the immediate effects of CA1 astrocytic modulation on neuronal activity during memory acquisition. GFAP::hM4Di mice were injected with saline or CNO before FC acquisition, and brains were collected 90-min later (Fig. 3a). CNO administration had no effect on HC–saline (*P < 0.05) (Fig. j). The average responses (k) from one mouse under saline and then under CNO are presented (average in a bold line, s.e.m. in shadow, blue light illumination in semitransparent blue). k. A downstream response of CA1 activation by Schaffer collateral optogenetic stimulation was detected in the ACC. The mean absolute value of the complex ACC response was found to have a significantly smaller amplitude under G_i pathway activation by CNO in CA1 astrocytes (n = 4 mice; *P < 0.05) (Fig. i). The average responses (l) from one mouse under saline and then under CNO are presented (average in a bold line, s.e.m. in shadow). Scale bars, 50 μm (applicable to all images).
the ACC, the BLA, the DG and the RSC. FC acquisition induced an overall increase in c-Fos expression in the CA1, the ACC and the BLA ($F_{(1,13)} = 8.1, P = 0.01$; $F_{(1,17)} = 5.07, P = 0.038$; $\bar{F}_{(1,13)} = 9.07, P = 0.008$, respectively), but not in the DG or the RSC (Fig. 3b–d; Extended Data Fig. 5b–h). Astrocytic manipulation in the CA1 did not substantially affect local neuronal c-Fos expression in this region in either home-caged or fear-conditioned mice (Fig. 3b,c; Extended Data Fig. 5c). To verify that the increase in c-Fos in the ACC following acquisition does not represent astrocytic activation in this region, we co-stained for c-Fos and GFAP and found only a negligible amount of cFos-expressing astrocytes (Extended Data Fig. 5i).

Surprisingly, $G_i$ activation in CA1 astrocytes substantially reduced the learning-induced elevation in c-Fos expression in the ACC, where no direct manipulation took place (Fig. 3b,d; Extended Data Fig. 5d). This result was reflected by a significant treatment by behavior interaction ($F_{(1,17)} = 5.04, P < 0.05$; FC-saline versus FC–CNO post-hoc, $P < 0.05$). The effect was specific to the ACC and not observed in other non-manipulated regions such as the BLA, the DG or the RSC (Extended Data Fig. 5e–h).

The finding that astrocytic $G_i$ pathway activation in the CA1 prevented the recruitment of the ACC during learning suggests a functional CA1→ACC connection, which can be modulated by hippocampal astrocytes. The existence of a monosynaptic CA1→ACC projection had been demonstrated\(^1^\), and a functional connection was reported using electrical stimulation\(^1^\). To generate synaptic ACC connection, which can be modulated by hippocampal astrocytes, we prevented the recruitment of the ACC during learning suggests a functional CA1→ACC connectivity, this finding and tissue damage selectively the activity of CA1 neurons projecting to the ACC, resulting in a significant effect on ACC activity, but only a moderate influence on total CA1 activity.

$G_i$ activation in CA1 astrocytes during memory acquisition specifically prevents the recruitment of CA1 neurons projecting to the ACC. From our findings that $G_i$ activation in CA1 astrocytes during learning prevents the recruitment of the ACC, and that CA1 astrocytes are able to modulate CA1→ACC functional connectivity, we drew the hypothesis that astrocytic $G_i$ activation can selectively prevent the recruitment of CA1 neurons projecting to the ACC, without similarly affecting other CA1 neurons.

To directly test this hypothesis, we tagged these projection neurons, measured their recruitment during memory acquisition and how it is affected by astrocytic $G_i$ activation. Mice were bilaterally injected with a retro-AAV inducing the expression of the Cre recombinase in excitatory neurons (AAV-retro-CaMKII::Cre) into the ACC and with a Cre-dependent virus inducing the expression of green fluorescent protein (GFP) (AAV5-ef1::DIO–GFP) into the CA1. AAV8-GFAP::hM4Di–mCherry was simultaneously injected into the CA1 to allow astrocytic manipulation (Fig. 4a). Together, these three vectors induced the expression of GFP only in CA1 neurons projecting to the ACC and of hM4Di in hippocampal astrocytes (Fig. 4b,c). These mice were injected with saline or CNO 30 min before FC acquisition or in their home cage, and brains were collected 90 min later. As in the previous experiment, CNO administration had no effect on immediate freezing following shock administration, and FC acquisition induced an overall increase in c-Fos expression in the CA1 ($F_{(1,13)} = 12.9, P = 0.002$). Moreover, astrocytic modulation was not sufficient to significantly reduce c-Fos expression in the CA1 (Fig. 4d,e). Furthermore, as before, modulation of CA1 astrocytes significantly reduced the learning-induced elevation in c-Fos expression in the ACC ($t_{(19)} = 2.6, P = 0.04$) (Fig. 3j,l). Astrocytic manipulation in the CA1 had a dramatic effect on the downstream response in the ACC to stimulation of the Schaffer collaterals as reflected by significantly attenuated field excitatory postsynaptic potentials (fEPSPs) following CNO administration (paired $t_{(9)} = 3.8, P = 0.01$) (Fig. 3k,l).

These results suggest that astrocytic manipulation in the CA1 can indeed modulate functional connectivity from the CA1 to the ACC.

We showed that $G_i$ pathway activation in CA1 astrocytes during fear memory acquisition prevents the recruitment of the ACC, without having a significant effect on local neuronal activity in the CA1, and that CA1 astrocytes can indeed modulate the functional CA1→ACC connectivity. These findings suggest that astrocytic manipulation selectively blocks the activity of CA1 neurons projecting to the ACC, resulting in a significant effect on ACC activity, but only a moderate influence on total CA1 activity.

**Fig. 4** $G_i$ pathway activation in CA1 astrocytes during memory acquisition specifically prevents the recruitment of CA1 neurons projecting to the ACC. a, Schematic of the experiment: AAV-retro-CaMKII::Cre was injected into the ACC, and AAV5-ef1::DIO–GFP together with AAV8-GFAP::hM4Di–mCherry were injected into the CA1. b, Together, these three vectors induced the expression of GFP (green) in CA1 neurons projecting to the ACC and hM4Di (red) in CA1 astrocytes. c, GFP-positive axons of CA1 projection neurons are clearly visible in the ACC. d, Mice expressing GFP in ACC-projecting CA1 neurons and hM4Di in their CA1 astrocytes that were injected with CNO ($n = 8$) 30 min before FC showed similar immediate freezing following shock administration. e, Fear-conditioned mice showed increased c-Fos levels in the ACC and with a Cre-dependent virus inducing the expression of green fluorescent protein (GFP) (AAV5-ef1::DIO–GFP) into the CA1. AAV8-GFAP::hM4Di–mCherry was simultaneously injected into the CA1 to allow astrocytic manipulation (Fig. 4a). Together, these three vectors induced the expression of GFP only in CA1 neurons projecting to the ACC and of hM4Di in hippocampal astrocytes (Fig. 4b,c). These mice were injected with saline or CNO 30 min before FC acquisition or in their home cage, and brains were collected 90 min later. As in the previous experiment, CNO administration had no effect on immediate freezing following shock administration, and FC acquisition induced an overall increase in c-Fos expression in the CA1 ($F_{(1,13)} = 12.9, P = 0.002$). Moreover, astrocytic modulation was not sufficient to significantly reduce c-Fos expression in the CA1 (Fig. 4d,e). Furthermore, as before, modulation of CA1 astrocytes significantly reduced the learning-induced elevation in c-Fos expression in the ACC ($t_{(19)} = 1.78, P = 0.049$) (Fig. 4e).

When specifically observing the subpopulation of CA1 neurons projecting to the ACC, these cells were dramatically recruited during memory acquisition, and astrocytic modulation significantly reduced the learning-induced cFos elevation in this population.

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(Fig. 4f). Specifically, in saline-treated mice, >15% of CA1→ACC cells expressed c-Fos following learning, whereas in CNO-treated GFAP::hM4Di mice, <5% CA1→ACC cells were active after learning, a level as low as that of home-caged mice (Fig. 4f–h; Extended Data Fig. 6a,b). This effect resulted in a significant treatment by behavior interaction ($F_{(1,21)} = 6.67, P = 0.017$; FC–saline versus FC–CNO post-hoc $P = 0.001$).

Finally, to test the specificity of our findings, we similarly tested an additional monosynaptic projection from the CA1 terminating at the nucleus accumbens (NAc). Mice were bilaterally injected with AAV-retro-CaMKII::iCre into the NAc, together with AAV5-ef1α::DIO–GFP and AAV8-GFAP::hM4Di–mCherry into the CA1, to tag CA1 neurons projecting to the NAc, and activated the G$_i$ pathway in CA1 astrocytes (Fig. 4i–k). As in the previous experiment, CNO administration before FC acquisition had no effect on immediate freezing (Fig. 4l). Activity in the NAc increased following FC ($F_{(1,22)} = 4.37, P = 0.048$), but, importantly, modulation of CA1 astrocytes had no effect on c-Fos expression after learning in
this region (Fig. 4m; Extended Data Fig. 6d,f). When we specifically tested c-Fos expression in the subpopulation of NAc-projecting CA1 neurons, we found that these neurons are only moderately recruited by learning ($F(1,23) = 4.41, P < 0.047$), and that astrocytic modulation had no effect on their activity (Fig. 4n; Extended Data Fig. 6c,e).

To conclude, we found that G i pathway activation in CA1 astrocytes specifically prevents the exceptional recruitment of CA1→ACC-projecting neurons during memory acquisition. The fact that the inhibition of this projection is induced by the same manipulation that specifically impairs remote memory acquisition suggests that the activity of CA1→ACC neurons during memory acquisition is necessary for remote recall.

Specific inhibition of CA1 neurons projecting to the ACC impairs the acquisition of remote, but not recent, memory. To specifically manipulate CA1→ACC neurons, mice were bilaterally injected with AAV-retro-CaMKII::iCre into the ACC and with a Cre-dependent hM4Di virus (AAV5-ef1α::DIO–hM4Di–mCherry) into the CA1 (Fig. 5a). Together, these vectors induced the expression of hM4Di–mCherry (red) in CA1 neurons projecting to the ACC. b, Together, these vectors induced the expression of hM4Di–mCherry (red) in CA1 neurons projecting to the ACC. c, hM4Di–mCherry-positive axons of CA1 projection neurons are clearly visible in the ACC. d, Mice expressing hM4Di in their ACC-projecting CA1 neurons were injected with either saline ($n = 9$) or CNO ($n = 9$) 30 min before FC acquisition. CNO application before training had no effect on baseline freezing (left) before shock administration or on recent contextual freezing (middle) the next day, but induced a significant decrease ($^* P < 0.05$) 20 days later compared with saline-treated controls (right). e, Active neurons expressing c-Fos were quantified in the CA1 and ACC regions. Impaired remote recall was accompanied by a reduced number of c-Fos-expressing neurons in the CA1 and the ACC ($^* P < 0.05$ for both). f, CNO administration reduced the recruitment of CA1→ACC cells during remote recall ($^* P < 0.03$; see e for the color key). g,h, Representative images of hM4Di (red) and c-Fos (green) in the CA1 (g) and ACC (h). Scale bars, 100 μm (applicable to all images). Data are presented as the mean ± s.e.m.
contextual memory task, as no effect was observed when the same mice were tested for auditory-cued memory in a novel context. That is, both groups demonstrated similar freezing in response to the tone 1 day after training and 20 days later (F(1,16) = 147.8, P = 1.7 × 10−3; F(1,16) = 37.8, P = 1.4 × 10−3, time main effect, respectively) (Extended Data Fig. 7b,c).

Finally, to gain insight into changes in neuronal activity accompanying this impaired remote retrieval of memories acquired under CA1→ACC-projection inhibition, brains were collected 90-min after remote recall and stained for c-Fos. We found that the impaired remote memory was accompanied by reduced c-Fos expression in both the CA1 (t(14) = −2.2, P = 0.022) and the ACC (t(14) = −2.4, P = 0.015) regions (Fig. 5e,g,h). When specifically observing the CA1→ACC neurons manipulated 3 weeks earlier, we found significantly reduced c-Fos expression (t(14) = −2, P = 0.033) (Fig. 5i,j).

In this experiment, we directly proved the involvement of CA1→ACC neurons in establishing the foundation for remote memory during acquisition, as suggested by the effect of astrocytes on this process.

Discussion

Recent years have seen a burst in discoveries of hitherto unknown elaborate roles for astrocytes in the modulation of neuronal activity and plasticity1. In this work, we showed that these cells can confer specific effects on neurons in their vicinity based on the distant projection target of these neurons. Specifically, astrocytic Gi activation during memory acquisition impairs remote, but not recent, memory retrieval. Another novel finding we presented is a massive recruitment of ACC-projecting CA1 neurons during memory acquisition, a process that is specifically inhibited by astrocytic manipulation, thus preventing successful recruitment of the ACC during learning. Finally, we directly inhibited this projection to prove its necessity for the formation of remote memory.

Chemogenetic and optogenetic tools, which were originally developed for use in neurons and allow real-time, reversible cell-specific manipulation, are now integrated into astrocyte research. Chemogenetic tools recruit intracellular pathways in astrocytes to induce clear behavioral effects, which greatly vary depending on the modulated cellular mechanisms12,14,15,22. For example, in our hands, Gi pathway activation in astrocytes (via Hm3Dq) leads to recent memory enhancement1, whereas in this work, we report that Gi pathway activation has no effect on recent memory and specifically impairs remote memory. In contrast to these clear differences in the downstream physiological and behavioral effects of astrocytic manipulation, the intracellular calcium dynamics recorded in astrocytes in reaction to very different stimuli are very much alike. For example, despite the fact that the Ga and Gi pathways are endogenously recruited by the administration of different neurotransmitters (for example, Ga by acetylcholine and Gi by GABA), c-Fos expression in astrocytes is similarly induced by activating either of these pathways13,14,20 (see also Fig. 1c), making it a good indicator for the occurrence of a modulation but not to its precise nature. Similarly, chemogenetic activation of either the Ga or the Gi pathway induced an increase in intracellular calcium in astrocytes11,14,19,22. However, whereas Ga pathway activation results in a long-lasting increase of Ca2+ activity11, we found that the effect of Gi pathway activation wanes in time, and on a behaviorally relevant time scale, even decreases slightly. The discrepancy between the clear downstream functional differences of astrocytic modulation by Ga and Gi DREADDs, and the similarity in calcium responses to these stimuli, may be resolved in the future by advanced imaging and analysis methods to provide insight to the intricacies of calcium signals in these cells.

Previous evidence suggests that astrocytes can have projection-specific effects based on either the input source or the output target of their neighboring neurons, but with some caveats. For example, in the central amygdala, astrocytic activation depressed inputs from the BLA and enhanced inputs from the central-lateral amygdala31. However, since the former projection is excitatory and the latter inhibitory, this finding could reflect specificity to the secreted neurotransmitter rather than to the projection source. Additionally, astrocytes in the striatum specifically modulate either the direct or indirect pathways6. Nonetheless, since the populations of striatal neurons from which these two projections originate differ genetically (expressing either the D1 or D2 dopamine receptors), it is impossible to determine whether the specificity that astrocytes demonstrate stems from surface protein expression in these neurons or their projection target. Similarly, astrocytes in the DG may differentially affect input from the medial perforant path, but the terminals of this pathway differ from the lateral perforant path in their exclusive expression of the GluN3a NMDA subunit31. Here, we showed that the differential effects of astrocytic modulation on CA1 pyramidal cells are based exclusively on their projection target. These cells may differ from other CA1 cells in the configuration of input they receive, in their activity pattern and possibly even in hitherto unidentified genetic properties.

The leading hypothesis in the memory field is that the hippocampus has a time-limited role in memory in that it is required for acquisition and recent recall, but becomes redundant for remote recall, being replaced by frontal cortices5. However, this temporal separation between the hippocampus and frontal cortex is not so rigid. For example, we and others have shown that the hippocampus is still critically involved in the consolidation and retrieval of remote memory (for examples, see refs. 33,35,36). Current research now attempts to define the temporal dynamics in different brain regions underlying remote memory23–25. The evidence regarding the role of frontal cortices during acquisition is mixed. For example, inhibition of medial entorhinal cortex input into the prefrontal cortex (PFC) during acquisition specifically impaired remote memory25. Conversely, inhibition of the PFC during acquisition had no effect on remote recall, nor did activation during remote recall of PFC neurons that were active during acquisition37. The role of the ACC in remote memory retrieval was repeatedly demonstrated by the finding that ACC inhibition during recall impairs remote but not recent memory in multiple tasks23–25,36,38. However, the time point at which the ACC is recruited to support remote memories was never defined. Here, we showed that the ACC is recruited at the time of initial acquisition, but the significance of this early activity is only revealed at the remote-recall time point. We further demonstrated that there is massive recruitment of ACC-projecting CA1 cells during learning, and that specific inhibition of this projection at this time point by astrocytes prevents the engagement of the ACC during acquisition, which results in impaired remote (but not recent) memory. When nonspecific CA1 inhibition is induced by direct neuronal Gi pathway activation, both recent and remote memory are impaired.

In this work, we revealed another novel capacity of astrocytes: they affect their neighboring neurons based on their projection target. This finding further expands the repertoire of sophisticated ways by which astrocytes shape neuronal networks and consequently high cognitive function.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-020-0679-6.

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Astrocytes were imaged three times for 3 min separated by a 1-min interval to determine baseline Ca
dlevels and activity. CNO or ACSF was then added to the chamber, and imaging (three times for 3 min separated by a 1-min interval) was resumed after a 10-min break. Signal processing and analysis were conducted using ImageJ (NIH) and Matlab. Temporal series were imported into ImageJ, and then astrocytic somas and their main branches were identified by their GCaMP6f fluorescence signals. We calculated the mode for each 3-min imaging epoch per ROI, then averaged the three epochs before and three epochs after the addition of CNO or ACSF. To quantify Ca events, we computed the integral of the Z score for each 3-min imaging epoch from the fluorescence (F) signal in each ROI. The Z score was calculated as 
\[ Z = (F - \mu) / \sigma \]
where \( \mu \) and \( \sigma \) are the mean and standard deviation, respectively, defined from the baseline activity of each cell (P < 0.05 percentiles). Negative Z scores were then averaged the Z scores of the three epochs before and three epochs after the addition of CNO or ACSF. Epochs for which the F signal throughout the 3 min had standard deviation values lower than 1 were assigned a Z score of 0 for the entire epoch. ROIs for which no active epochs were detected before and after manipulation were excluded from the analysis.

**Immunohistochemistry.** Three weeks post-injection, mice were transcardially perfused with cold PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were extracted, postfixed overnight in 4% PFA at 4°C and cryoprotected in ascending concentrations of sucrose (20–30% in PBS) for 24 h. Tissue was sectioned to a thickness of 40 μm using a sliding freezing microtome (Leica SM 2010R) and preserved in a cryoprotectant solution (25% glycerol and 30% ethylene glycol in PBS). Free-floating sections were washed in PBS, incubated for 1 h in blocking solution (1% BSA and 0.3% Triton X-100 in PBS) and incubated overnight at 4°C with primary antibodies (see below for a full list of antibodies) in blocking solution. For the c-Fos staining, slices were incubated in blocking antibody for 1 h at room temperature. After washed with PBS and incubated for 2 h at room temperature with secondary antibodies (see below for a full list of antibodies) in 1% BSA in PBS. Finally, sections were washed in PBS, incubated with a 46-diamidino-2-phenylindole (DAPI, 1 μg ml\(^{-1}\)) and mounted on slides with mounting medium (Fluoromount-G, eBioscience). For neurogenesis staining, BrdU (Sigma, 100 μg per kg) was injected i.p. together with the CNO injection and 2 h after the FC training. At 90 min after recent or remote recall, brains were removed and slices prepared as described above. Sections were fixed in 50% formamide and 50% SSC for 2 h at 65°C, then incubated in 2 N HCl for 30 min at 57°C and neutralized in boric acid for 10 min. After de-washes, sections were blocked in 1% BSA with 0.1% Triton-X for 1 h at room temperature. Sections were incubated in anti-BrdU for 48 h at 4°C. Sections were then washed with PBS and incubated with a secondary antibody for 2 h at room temperature.

**Antibodies. Primary antibodies.** The following primary antibodies were used: anti-GFAP (Millipore, catalog no. AB5804; diluted 1:500); rabbit anti-NeuN (Cell Signaling Technology, catalog no. 12343; diluted 1:400); rat anti-BrdU (Bio-Rad, catalog no. 010303G; diluted 1:200); guinea pig anti-DCX (Millipore, catalog no. AB2253; diluted 1:10,000); and rabbit anti c-Fos (Sypnic Systems, catalog no. 226 003; diluted 1:10,000).

**Secondary antibodies.** The following secondary antibodies were used, all from Jackson Laboratories: donkey anti-chicken (conjugated to Alexa Fluor 488, catalog no. 703-545-155; diluted 1:500); donkey anti-rabbit (conjugated to Alexa Fluor 488, catalog no. 711-545-152; diluted 1:500); donkey anti-goat (conjugated to Alexa Fluor 594, catalog no. 706-605-148; diluted 1:100); and donkey anti-rat (conjugated to Cy3, catalog no. 706-605-148; diluted 1:100); and donkey anti-rat (conjugated to Cy5, catalog no. 711-545-152; diluted 1:100).

**Confocal microscopy.** Confocal fluorescence images were acquired on an Olympus scanning laser microscope (Fluoview FV1000) using x4 and x10 objectives. The laser sources were transformed using either ImageJ (NIH) or Flouview Viewer v4.2 (Olympus). Cells were counted in a blinded manner.

**Behavioral testing.** The FC apparatus consisted of a conditioning box (18 x 18 x 30 cm), with a grid floor wired to a shock generator surrounded by an acoustic chamber (Ugo Basile) and controlled by EthoVision software (Noldus). Three weeks after injections, mice were placed in the conditioning box for 2 min, and then a pure tone (2.9 kHz) was sounded for 20 s followed by a 2-s foot shock (0.4 mA). This procedure was then repeated, and 30 s after the delivery of the second shock, mice were returned to their home cages. FC was assessed through the continuous measurement of freezing (complete immobility), which is the dominant behavioral fear response. Freezing was automatically measured throughout the testing trial using EthoVision tracking software. To test contextual FC, mice were placed in the original conditioning box, and freezing was measured for 5 min. To test auditory-cued FC, mice were placed in a different context (a cylinder-shaped cage with stripes on the walls and a smooth floor), freezing was then measured for 5 min.
was measured for 2.5 min and then a 2.9-kHz tone was sounded for 2.5 min, during which conditioned freezing was measured. Mice were tested for recent memory 24 h after acquisition and for remote memory 21 or 28 days later. In one experiment, an additional remote memory test was performed 66 days after acquisition.

The NAPR test was conducted in a round plastic arena (54 cm in diameter) or a square or a trapezoid arena with an identical area size (2,290 cm²). Mice were placed in the center of the arena and allowed to freely explore for 5 min. Habituation to the familiar environment (reduced exploration between first and second exposures) was measured using EthoVision tracking software.

CNO (Tocris) was dissolved in dimethylsulfoxide (DMSO) and then diluted in 0.9% saline solution to yield a final DMSO concentration of 0.5%. Saline solution for control injections also consisted of 0.5% DMSO. CNO (100 mg per kg) was i.p. injected 30 min before the behavioral assays. In the relevant experiments, BrdU (Sigma, B502; 100 mg per kg) was injected i.p. together with the CNO or saline and 2 h after the behavioral experiment.

In vivo electrophysiology and optogenetics. Simultaneous optical stimulation of the Schaffer collaterals and electrical recordings in the CA1 and the ACC were performed as follows. Mice were anesthetized with isoflurane, and an optrode (an extracellular tungsten electrode (1 MΩ, ~125 μm) glued to an optical fiber (200-μm core diameter, 0.39 NA) with the tip of the electrode protruding ~400 μm beyond the fiber end) was used to record local field potentials in the stratum radiatum and to illuminate the Schaffer collaterals. fEPSP recordings were conducted with the optrode initially placed above the dorsal CA1 (AP: −1.6 mm; ML: 1.1 mm; DV: −1.1 mm) and gradually lowered in 0.1-mm increments into the stratum radiatum (~1.55 mm). The optical fiber was coupled to a 473-nm solid-state laser diode (Laserglow Technologies) with ~10 mW of output from the fiber. fEPSP recordings from the ACC were similarly performed using an extracellular tungsten electrode (1 MΩ, ~125 μm) placed over the ACC (AP: 0.25 mm; ML: 0.4 mm; DV: −1.3 mm) and gradually lowered in 0.1-mm increments to 1.8 DV. This electrode was dipped in DiI (1 mg per 1.5 ml in 99% ethanol; Invitrogen) to validate the position of the recording site.

To optogenetically activate the Schaffer collaterals, blue light (473 nm) was unilaterally delivered through the optrode. The photostimulation duration was 10 ms, delivered 72 times for each treatment (saline or CNO) every 5 s. Saline or CNO were injected i.p., and recording was started 30 min after each injection.

Recordings were carried out using a Multiclamp 700B patch-clamp amplifier (Molecular Devices). Signals were low-pass filtered at 5 kHz, digitized and sampled through an AD converter (Molecular Devices) at 10 kHz and stored for offline analysis using Matlab (Mathworks). CA1 responses to Schaffer collateral stimulation were quantified by calculating the amplitude of the fEPSPs relative to the mean baseline levels, defined as a 200-ms time window before photostimulation. CA1 activation by Schaffer collateral stimulation resulted in a complex downstream activity in the ACC that lasted ~400 ms. Because this signal had both positive and negative peaks, to estimate the overall magnitude of the response, we calculated its mean absolute value over the entire 400-ms period, from the beginning of photostimulation in the CA1.

Statistical analysis. The results of automatic or blind measurements were analyzed using two-way ANOVA followed by least significance difference post-hoc tests or using Student’s t-test, both one-sided, as applicable. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data used to support the conclusions of this study are publicly available at https://data.mendeley.com/datasets/9jw3kdhbb7/1, and as indicated in the Nature Research Reporting Summary. Source data are provided with this paper.

Code availability
Analysis codes will be made available to any interested reader.

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Author contributions
A.K. performed all in vivo electrophysiology and two-photon calcium imaging experiments. A.A. contributed to the behavioral experiments. M.G. produced the AAV vectors. T.K. contributed to the behavioral experiments and performed all projection targeting and histology. M.L. co-supervised the electrophysiology experiments. I.G. conceived and supervised all aspects of the project, and wrote the manuscript with input from all other authors.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to I.G.
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Extended Data Fig. 1 | Prolonged Gi pathway activation in CA1 astrocytes reduces their calcium activity (Related to Fig. 1). Following an injection of AAV8-GFAP::hM4Di-mCherry, hM4Di was expressed in 87% (491/552 cells from 4 mice) of CA1 astrocytes (a), with >96% specificity (491/507 cells, from 4 mice) (b). c, d, Minimal co-localization with the neuronal nuclear marker NeuN was detected (scale bar 50 µm; 0.9% expression in neurons, 7/766 cells). (e, g) Representative astrocytes expressing GCaMP6f (white) and hM4Di-mCherry (not visible, but see Fig. 1d) were exposed either to ACSF (e, f) or CNO (g, h). Representative ROIs, and their activity in corresponding colors are presented. Scale bars = 100 µm. CNO application triggered a decrease in baseline intracellular Ca\(^{2+}\) levels, and reduced the total size of Ca\(^{2+}\) events in these cells (see Fig. 1f,g). Data presented as mean ± standard error of the mean (SEM).
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Astrocytic Gi activation in CA1 during learning had no effect on auditory-cued remote memory (Related to Fig. 1). GFAP::hM4Di mice were injected with Saline (n = 7) or CNO (n = 6) 30 min before Fear Conditioning (FC) acquisition. CNO application before training had no effect on exploration of the conditioning cage (a), or on auditory-cued memory recall either 24 hr after acquisition (b) or 20 days after that (c) in a novel context, with both groups showing increased freezing during tone presentation (p < 0.001, p < 0.01, respectively). d, Bilateral double injection of AAV5-CaMKIIα::hM4Di-mCherry resulted in hM4Di-mCherry expression in CA1 Neurons only (top). Scale bar - 100 μm. The groups did not differ in the percent of hM4Di-expressing cells level of expression (Saline - 20.5%, CNO - 20.6%; bottom). CaMKIIα::hM4Di mice were injected with either Saline (n = 9) or CNO (n = 10) 30 min before FC acquisition. CNO application before training had no effect on exploration of the conditioning cage (e), or on auditory-cued memory recall either 24 hr after acquisition (f) or 20 days after that (g) in a novel context, with both groups showing increased freezing during tone presentation (p < 0.000001, p < 0.00001, respectively). h, In a new group of GFAP::hM4Di mice, CNO administration (n = 12) only during the recall tests had no effect on either recent or remote memory, compared to Saline-injected controls (n = 12). In these mice, CNO administration during recall also had no effect on auditory cued memory either 24 hr after acquisition (i) or 20 days after that (j), compared to Saline-injected controls. When CNO was not administered during acquisition of the non-associative place recognition task, the GFAP::hM4Di mice (n = 6) from Fig. 1l showed equivalent performance to controls (n = 7; p < 0.01)(K). Example exploration traces and average Δ are shown (right). Data presented as mean ± SEM.
Extended Data Fig. 3 | CNO application itself during learning had no effect on remote memory (Related to Fig. 1). a, Bilateral double injection of AAV8-GFAP-eGFP resulted in eGFP expression in CA1 astrocytes only. Scale bar – left 300 µm, right 50 µm. Mice expressing eGFP in their CA1 astrocytes were injected with either Saline (n = 6) or CNO (n = 7) 30 min before fear conditioning acquisition. CNO administration before training to eGFP-expressing mice had no effect on baseline freezing or recent contextual memory recall one day later (A). Neither did CNO have any effect on remote memory 20 days later or 45 days after that (C). In the non-associative place recognition test, CNO application before a first visit to a new environment had no effect on remote memory 28 days later (D), reflected by a similar decrease (p < 0.0001) in the exploration between Saline injected (n = 6) and CNO-treated mice (n = 7) Example exploration traces and the average change (Δ) following treatment are shown on the right. Data presented as mean ± SEM.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | CNO administration during acquisition reduces CA1 and ACC activity at the time of remote recall only in GFAP::hM4Di mice, and does not affect neuronal proliferation, differentiation, or survival (Related to Fig. 2). a, Active neurons expressing cFos were quantified in the CA1, ACC, dentate gyrus (DG), retrosplenial cortex (RSC), and basolateral amygdala (BLA). GFAP::hM4Di mice from Fig. 2a, b that were injected with CNO (n = 6) before fear conditioning and showed impaired remote recall compared to Saline controls (n = 6), also demonstrated reduced number of cFos expressing neurons in CA1 and ACC (p < 0.05 for both) (b). No changes in cFos expression in the DG or RSC were observed in these mice, but the reduced fear was accompanied by a significant reduction in cFos expression in the BLA (p < 0.011) (c). GFAP::eGFP control mice were injected with CNO (n = 5) or Saline (n = 5) before fear conditioning, and then tested on the next day. No changes were observed in recent memory (d) or in the number of neurons active during recent recall in the CA1 or ACC (e). Other GFAP::eGFP mice were injected with CNO (n = 5) or Saline (n = 6) before fear conditioning, and then tested on the next day and again 21 days later. No changes were observed in recent or remote memory (f), or in the number of neurons active during remote recall in the CA1 or ACC (g). Representative images of GFAP::eGFP (green) and cFos (red in H,J green in I,K) following recent (h,j) or remote (j,k) recall in the CA1 (h,j) and ACC (i,k) are presented. I, GFAP::eGFP mice were injected with CNO or Saline together with BrdU before fear conditioning, and then tested on the next day. No changes were observed in stem cell proliferation (BrdU in white) (m) or in the number of young, Doublecortine (DCx)-positive neurons (white) (n). o, GFAP::eGFP mice were injected with CNO or Saline before fear conditioning, and then tested 21 days later. No changes were observed in stem cell proliferation and differentiation (p) or in the number of young, DCx-positive neurons (q). Scale bars = 100μm for CA1, ACC and whole DG, 10 μm for zoomed-in cells. Data presented as mean ± SEM.
Extended Data Fig. 5 | Gi pathway activation in CA1 astrocytes during memory acquisition does not affect the recruitment of the RSC and DG (Related to Fig. 3).

a, GFAP::hM4Di mice that were injected with CNO (n = 9) or Saline (n = 9) 30 minutes before fear conditioning showed similar immediate freezing following shock administration to Saline-injected controls. b, Active neurons expressing cFos were quantified in the CA1, basolateral amygdala (BLA), ACC, retrosplenial cortex (RSC) and dentate gyrus (DG) of GFAP::hM4Di mice that were injected with CNO (n = 9) or Saline (n = 9) 30 minutes before fear conditioning, or in home-caged mice (CNO n = 4, Saline n = 4). c, Representative images of hM4Di (red) and cFos (green) in the CA1 (c) and ACC (d) of home caged GFAP::hM4Di mice showing no effect of CNO administration on cFos levels. cFos-expressing astrocytes are observed below and above the CA1 pyramidal layer. Scale bars = 100 μm. d, Fear-conditioned GFAP::hM4Di mice showed increased cFos levels in the BLA compared to home-caged mice (p < 0.01), but CNO administration had no effect on either group. Fear-conditioning and CNO administration had no effect on cFos levels in the RSC and DG. Representative images of hM4Di (red) and cFos (green) in the BLA (f), RSC (g) and DG (h) are presented. i, Double staining for cFos and GFAP showed a negligible (0.34%) percent of ACC astrocytes that express cFos. j, An electrode dipped in Dil was placed in the ACC to record the response to CA1 activation. k, The location of the electrode in the ACC is shown in crimson, and no ChR2-eYFP positive axons (green) are observed in this region. All scale bars = 100 μm. Data presented as mean ± SEM.
Extended Data Fig. 6 | Gi pathway activation in CA1 astrocytes has no effect on cFos expression in home-caged mice (Related to Fig. 4). a, b, Representative images of hM4Di in astrocytes (red), GFP in ACC-projecting CA1 neurons (green) and cFos (pink) in the CA1 of Saline- (A) or CNO- (B) injected home-caged mice are presented. No effect of CNO on cFos levels was observed. c, e, Representative images of hM4Di in astrocytes (red), GFP in NAc-projecting CA1 neurons (green) and cFos (pink) in the CA1 of Saline- (C) or CNO- (E) injected fear-conditioned mice are presented, showing no effect of the astrocytic manipulation on CA1→NAc neurons activity. The GFP-positive axons of these CA1 neurons are clearly observed in the NAc (d, f), with no apparent effect on cFos expression in this region. All scale bars=50μm.
Extended Data Fig. 7 | Specific inhibition of CA1-to-ACC projection during learning had no effect on auditory-cued memory (Related to Fig. 5).

CA1→ACC-hM4Di mice were injected with Saline (n = 9) or CNO (n = 9) 30 min before FC acquisition. CNO application before training had no effect on exploration of the conditioning cage (a), or on auditory-cued memory recall either 24 hr after acquisition (b) or 20 days after that (c) in a novel context, with both groups showing increased freezing during tone presentation (p < 0.00001, p < 0.0001, respectively). Data presented as mean ± SEM.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- A description of all covariates tested
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- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) and variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | Behavioral data was collected using the EthoVision software (Noldus). |
|-----------------|---------------------------------------------------------------------|
| Data analysis   | Data was analyzed using Matlab and Fiji image J, statistical analysis was performed using the SPSS software. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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- A list of figures that have associated raw data
- A description of any restrictions on data availability

The datasets generated during the current study, and the analysis codes are available online.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Commonly used mouse sample sizes (5-10 for behavior, 4-5 for electrophysiology), which based on our previous experience are sufficient to reach statistical significance while keeping the number of animals to a minimum were used.

Data exclusions

Data was excluded only on technical basis (e.g. unsuccessful injection determined by expression imaging post-mortem, software failure during behavioral data collection or faulty electrodes during recordings).

Replication

The major result of the paper is replicated within it (see figures and Extended Data figures)

Randomization

Mice were randomly allocated into groups.

Blinding

All behavioral experiments and histological quantification were performed blindly.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|------------------------|
| [ ] | Antibodies             |
| [ ] | Eukaryotic cell lines   |
| [ ] | Palaeontology and archaeology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |
| [ ] | Clinical data           |
| [ ] | Dual use research of concern |

**Methods**

| n/a | Involved in the study |
|-----|------------------------|
| [ ] | ChIP-seq               |
| [ ] | Flow cytometry         |
| [ ] | MRI-based neuroimaging |

Antibodies

| Antibodies used | All details are provided in the methods section. |
|-----------------|-------------------------------------------------|
| Validation      | All details (a reference for every primary antibody) are provided in the methods section. |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

Male C57BL6 mice, 6-7 weeks old (Harlan)

**Wild animals**

The Study did not involve wild animals

**Field-collected samples**

The Study did not involve samples collected in the field

**Ethics oversight**

Experimental protocols were approved by the Hebrew University Animal Care and Use Committee and met guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.