

Optogenetic silencing hippocampal inputs to the retrosplenial cortex causes a prolonged disruption of spatial working memory

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Abstract

Summary

Working memory allows us to keep information in memory for the time needed to perform a given task. Such fundamental cognitive ability relies on a neural circuit including the retrosplenial cortex (RSC), connected to several cortical areas, functionally and anatomically, namely primary visual areas, and higher cognitive areas such as the cingulate, midcingulate, and subicular cortices. RSC bears intimate anatomical and functional connections with the hippocampus, and has been implicated in integrating and translating spatial-temporal contextual information between ego- and allocentric reference frames, to compute predictions about goals in goal-directed behaviors. The relative contribution of the hippocampus and retrosplenial cortex in working memory-guided behaviors remains unclear due to the lack of studies reversibly interfering with synapses connecting the two regions during such behaviors. We here used eArch3.0, a hyperpolarizing proton pump, to silence hippocampal axon terminals in RSC while animals perform a standard delayed non-match to place task. We found that such manipulation impairs memory retrieval, significantly decreasing performance and hastening decision-making. Furthermore, we found that such impairment outlasts light-activation of the opsin, its effects being noticed up to 3 subsequent trials.

eLife assessment

The authors report that optogenetic inhibition of hippocampal axon terminals in retrosplenial cortex impairs the performance of a delayed non-match to place task. Elucidating the role of hippocampal projections to the retrosplenial cortex in memory and decision-making behaviors is **important**. However, the strength of evidence for the paper's claims is **incomplete**.

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Introduction

The ability to guide behavior according to knowledge stored previously in memory lies at the heart of adaptive behavior, and is essential for survival. A crucial aspect of adaptive behavior is the capacity to make decisions based on the spatial structure of the surrounding context, something globally known as spatial cognition or spatial goal-directed behavior. Such cognitive function starts with the selection of relevant information from a flood of sensory stimuli, and ends with its storage in cortico-hippocampal circuits (Morris et al., 1982 [↗](#); Nadel, 1990 [↗](#); Scoville et al., 1957 [↗](#)) ultimately as a cognitive map manifested in a vast repertoire of neural activity, selective to multiple ego-(self-referenced) and allocentric (world-referenced) spatial variables, the seminal example of which is the existence of hippocampal place-selective neurons (O'Keefe and Dostrovsky, 1971 [↗](#)). To allow for the planning of the movements necessary to reach an intended goal, all the while ensuring that body actions are consistent with the context acted upon, a mechanism must exist that reinstates (retrieves) allocentric contextual representations, stored in the hippocampus (HIPP) as spatial memories, aligned to the animal's viewpoint, e.g. from an egocentric perspective (Bicanski and Burgess, 2018 [↗](#); Burgess, 2008 [↗](#); Gomez et al., 2009 [↗](#), 2013 [↗](#); Michelmann et al., 2020 [↗](#)). In such circuit, the retrosplenial cortex (RSC) emerges as a cornerstone, encoding contextual variables in both ego-and allocentric reference frames, representing future spatial goals, and effectively anchoring spatial memory-dependent decisions (Alexander and Nitz, 2015 [↗](#); Alexander et al., 2020 [↗](#); Burgess, 2008 [↗](#); Carstensen et al., 2021 [↗](#); Gomez et al., 2009 [↗](#), 2013 [↗](#); Michelmann et al., 2020 [↗](#); Nitzan et al., 2020 [↗](#)).

Despite this remarkable work, we are yet to determine which neural pathways convey allocentric-referenced information stored in the HIPP, through hypothetical recall and egocentric referencing in RSC, onto the anterior midline cingulate (CG) and medial pre-frontal cortical areas, responsible for planning motor behavior according to previous events, present needs, and motivation (Akam et al., 2020 [↗](#); Brown and Braver, 2005 [↗](#); Cowen et al., 2012 [↗](#); Jin et al., 2009 [↗](#); Miller et al., 2019 [↗](#); Sober and Sabes, 2003 [↗](#)). We have recently found that HIPP axons target the divisions of the medial mesocortex: CG, midcingulate and RSC, according to a gradient, in which RSC receives a combination of anatomically and functionally distinct inhibitory and excitatory functional synapses (Ferreira-Fernandes et al., 2019 [↗](#)), whose manipulation results in specific behavioral deficits (Yamawaki et al., 2019 [↗](#)). We hypothesize that these direct connections between hippocampal CA1 and RSC convey recently encoded and recalled memories to the relevant medial mesocortical regions.

To test this hypothesis we used the Delayed Non-Match to Place task (DNMP), a spatial working memory (WM) task that requires animals to retrieve the memory of a previous decision, and of the time it occurred, to disambiguate it from similar, previously rewarded, decisions (Aggleton et al., 1986 [↗](#); Maguire, 2001 [↗](#); Shaw and Aggleton, 1995 [↗](#); Shaw et al., 2013 [↗](#); Wilson et al., 2013 [↗](#)). We then used optogenetics to reversibly interrupt synaptic transmission between HIPP and RSC neurons during the retrieval phase of the DNMP task. Optogenetics uses light-activated membrane-bound molecules with the capacity to sustainedly, and reversibly, change neuronal membrane potential, through various molecular mechanisms, namely opening ion channels or pumping ions across the membrane (L Fénno, 2010 [↗](#)), to excite or silence groups of neurons with behavior-specific temporal accuracy. As far as it is known, optogenetics is the only molecular technique allowing for reversible perturbation of neural activity within the time constants of the cognitive functions at play during working memory (Yamamoto et al., 2014 [↗](#)).

To test the hypothesis that HIPP inputs onto RSC carry contextual information necessary for DNMP performance, and are thus necessary for spatial working memory, we used the optogenetic neural silencer eArch3.0 expressed in hippocampal neurons via local injection of an anterograde, static, viral vector (reviewed in (Saleeba et al., 2019 [↗](#))), AAV5-hSyn1-eArch3.0, under a pan-neuronal

promoter, or its “empty” counterpart with only YFP, in adult male Long Evans rats previously trained in DNMP to 75% performance. We then ran multiple 28-trial DNMP sessions, in which we randomly interleaved two types of trials: no-illumination (NI) or illumination during the Test, or retrieval, phase (TI), with green (525 nm) light delivered via 2 diagonal, intra-cerebral lambda fiberoptic implants, longitudinally staggered such as to deliver light to the full extent of RSC. In each animal we measured three performance variables: individual trial (error/correct) and global performance (% correct trials), response latency to the choice point (CP), and time spent at the CP. We then used multivariable Generalized Linear Mixed Model (GLMM) using the above categorical and continuous variables, to investigate the impact of silencing hippocampal synapses onto RSC neurons in spatial WM-dependent behavior.

If indeed the HIPPO input onto RSC neurons anchors the decisions during “Test” runs to the memory of the “Sample” runs, we predict that by silencing HIPPO inputs in RSC we will prevent the transfer of information previously encoded in HIPPO, and thus hinder the mechanism allowing RSC to inform the correct trajectory during the “Test” run, resulting in lower performance, specifically in eArch+ animals.

Results

Injection of AAV-5-hSyn1-eArch3.0 results in significant expression of a fluorescent tag in RSC hippocampal terminals

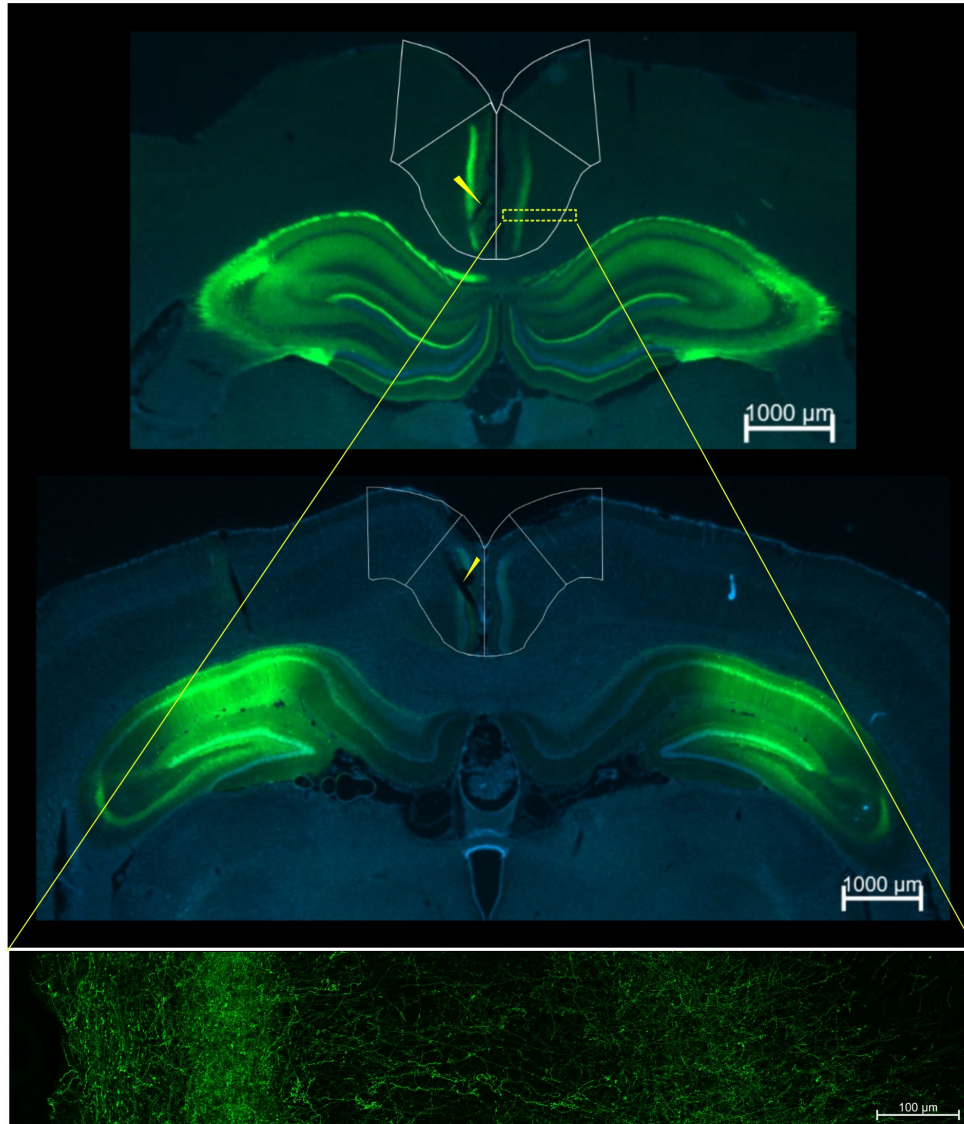
Besides abundant expression of both eArch and CTRL transgenes in hippocampal CA1, we found highly significant expression in the RSC layers as previously reported ([Figure 1A](#) [↗](#), and S1) (Ferreira-Fernandes et al., 2019 [↗](#)). We could observe that HIPPO axon terminals are distributed with high density in the superficial RSC layers II-III, as well as in deeper layer V, and sparsely on all other layers, forming beads-on-a-string structures, typical of axon terminals establishing synapses. On these RSC regions we surgically implanted two fiberoptic stubs diagonally to target both hemispheres, at a relative reverse angle in distinct AP coordinates ([Figure 1B](#) [↗](#), Methods).

DNMP sessions in which no light was delivered resulted in no differences in behavioral performance between eArch+ and CTRL animals

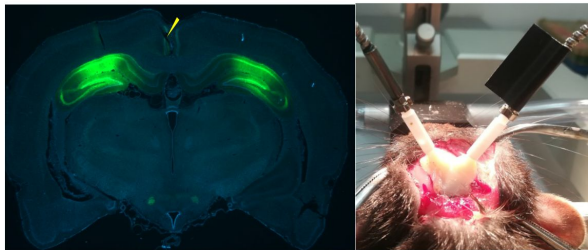
Freely moving behavior is conditioned by the relations of body shape and movement, with surrounding context structure and dynamics. As such, to make sure all behavioral alterations observed during light-delivery and opsin activation are due solely to this mechanism, it is imperative that pre-illumination DNMP testing is run under the same conditions as those used while we silence RSC-HIPPO axon terminals, except for light delivery. The DNMP task includes a sample (encoding) phase, a delay (memory retention) period, and a choice (retrieval) phase, on a T-shaped maze apparatus. During the sample run, animals are rewarded for running the only available arm, with the alternative arm being blocked. This is followed by the delay period, and the “Test” (retrieval) run, during which both arms are made available, and animals are rewarded for choosing the arm previously blocked (i.e., “not matching” the arm sampled previously).

We thus ran the DNMP protocol in two groups of animals expressing either AAV-5-hSyn1-eArch3.0 (eArch+) or an empty construct (CTRL) AAV-5-hSyn1-YFP, with fiberoptic stubs surgically implanted, and patch cords attached to the fiberoptic stubs, no light being delivered ([Figure 2 A-B](#) [↗](#)). Under such conditions we found no differences between eArch+ and CTRL animals in either of the three sessions ran ([Figure 2C](#) [↗](#), Table 1, GLMM, $p=0.212$, Bonferroni *post-hoc* test).

A



B



C

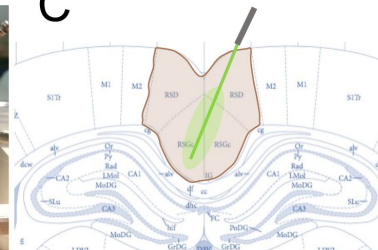


Figure 1.

Viral vector expression and fiberoptic placement

(A) Anatomical distribution of eYFP fluorescence expression in distinct AP coordinates of the retrosplenial cortex after injection in the hippocampus (HIPP). (B) Injection spots in the HIPP (C) Fiberoptic placement two fiberoptic stubs at AP reversed 30 ° angles reaching the two hemispheres at two distinct AP levels of RSC (arrows point to fiberoptic tracks, please refer to Figure S1 for low-mag images of all animals and examples of high-mag images).

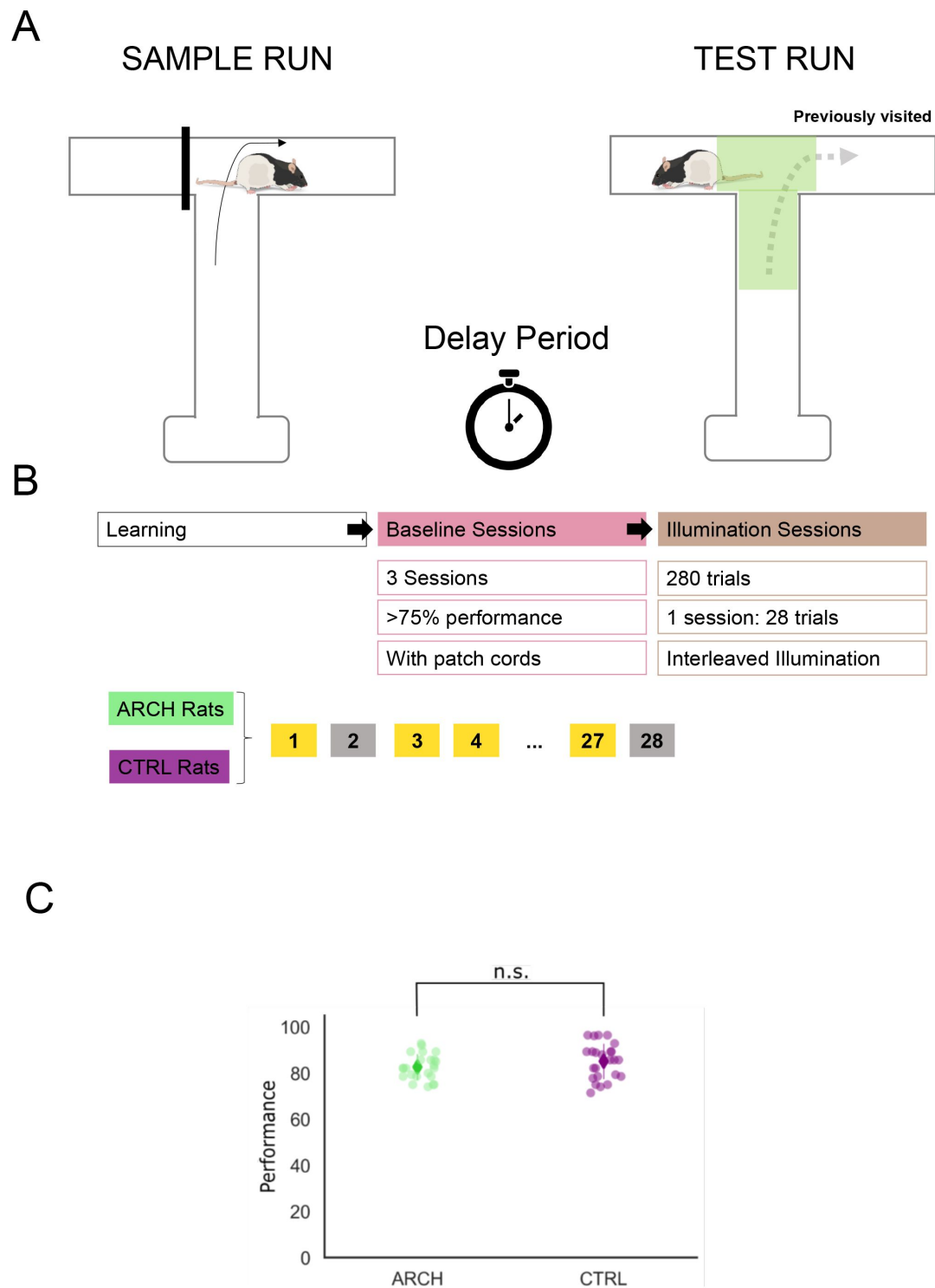


Figure 2.

Delayed Non-Match to Trajectory protocol and baseline performance

(A) DNMP protocol. Rats explore the unblocked arm in a sample run, then wait in the starting area for a 15 sec delay. Once back on the track, with both arms unblocked, rats receive a reward if they choose the arm opposite the one explored in the sample run. (B) Training schedule, after reaching 75% performance, 3 baseline (no light delivered) sessions are followed by 10 sessions with illuminated trials interleaved with non-illuminated ones. (C) No differences in performance were observed in the baseline sessions (GLMM, $p=0.212$, Bonferroni post-hoc test)

eArch activation via green light illumination resulted in significantly lower global performance in eArch+, but not CTRL, animals

After the “baseline” phase above, we started the “illumination” phase protocol, in which “Test-illuminated” trials (TI), with light delivered through the fiberoptic implants in the Test epoch of the DNMP protocol, were randomly interleaved with “non-illuminated” trials (NI), where no light was delivered in the same ~25 min session (**Fig. 2B**). In these sessions we observed a significant difference between eArch+ and CTRL animals, with an overall lower performance in eArch+ animals (**Fig. 3A**, Table 2, GLMM, $p=0.006$, Bonferroni *post-hoc* test). In addition, we found that the ratio of correct trials in the TI and NI trial groups (**Figure 3B**, Tables 2 and 3), were both significantly different from baseline levels ($p<0.001$), and from their CTRL counterparts (TI, $p=0.02$, NI, $p=0.008$), whose performance did not differ from baseline ($p=0.252$, $p=0.184$). The fact that more errors were committed in both TI and NI trials of an illuminated session, but only in eArch+ animals, being absent from CTRL animals, implies two things: first, the effect we observe is not the result of light alone acting upon neurons, an effect described earlier in striatal medium spiny (Owen et al., 2019), and CG neurons (Stujenske et al., 2015); second, the effects of eArch light activation of hippocampal inputs onto RSC neurons *outlasted illumination*, affecting subsequent NI trials. We were surprised by this result, as it indicates that the effects of eArch activation on either, or both, the neurophysiology of HIPP-RSC synapses and the ensuing circuit mechanisms involved in DNMP, last longer than would be expected. To directly address this possibility, we analyzed the probability of animals committing errors after illuminated trials. We observed that, overall, illuminated trials were followed by a decreased probability of correct trials on eArch+, but not CTRL, animals (**Fig. 3C**, Table 4, $p=0.015$). This is consistent with the effects of eArch activation outlasting illumination by enough time such as to affect subsequent trials.

The effect of light delivery in subsequent non-illuminated trials in sessions where the two types of trials are interleaved within 1-2 minutes, as in the present study, has at least one precedent study in (Robinson et al., 2017). In that study, trials in which a silencer opsin is activated by light are characterized by increased error probability, and abnormal neural activity with decreased temporal selectivity and coding. Thus, both neural activity and behavior of interleaved non-illuminated trials are affected by the activation of the opsin in illuminated trials, leading authors to hypothesize that such persistent disruption of temporal coding in light-off trials is caused by synaptic plasticity events largely specific to the neural activity taking place in the (previous) epoch where light is delivered.

Our findings also imply that the notion of millisecond time-resolved neuron silencing using eArch is not applicable to synaptic transmission and, by consequence, to the ensuing behavioral effects we now investigate.

Contrary to CTRL animals, eArch+ animals do not correct course following an incorrect decision, in illuminated sessions

A fundamental principle of learning theory, and a common empirical finding in reward-based learning experiments, is that following an error in which reward is withheld, animals will correct their course of action. Conversely, following a correct decision, which is rewarded, animals maintain their course of action. This behavior is presumably informed by the association of the previous action with resulting outcome, which they store in memory for the next trial (Mizumori and Jo, 2013). Under this assumption, we predict that in eArch+, but not CTRL animals, illuminated trials will be followed by persistently defective HIPP-RSC synaptic function, presumably preventing subsequent decisions from anchoring themselves to the previous action-outcome association, even in the absence of light delivery, resulting in a higher probability of

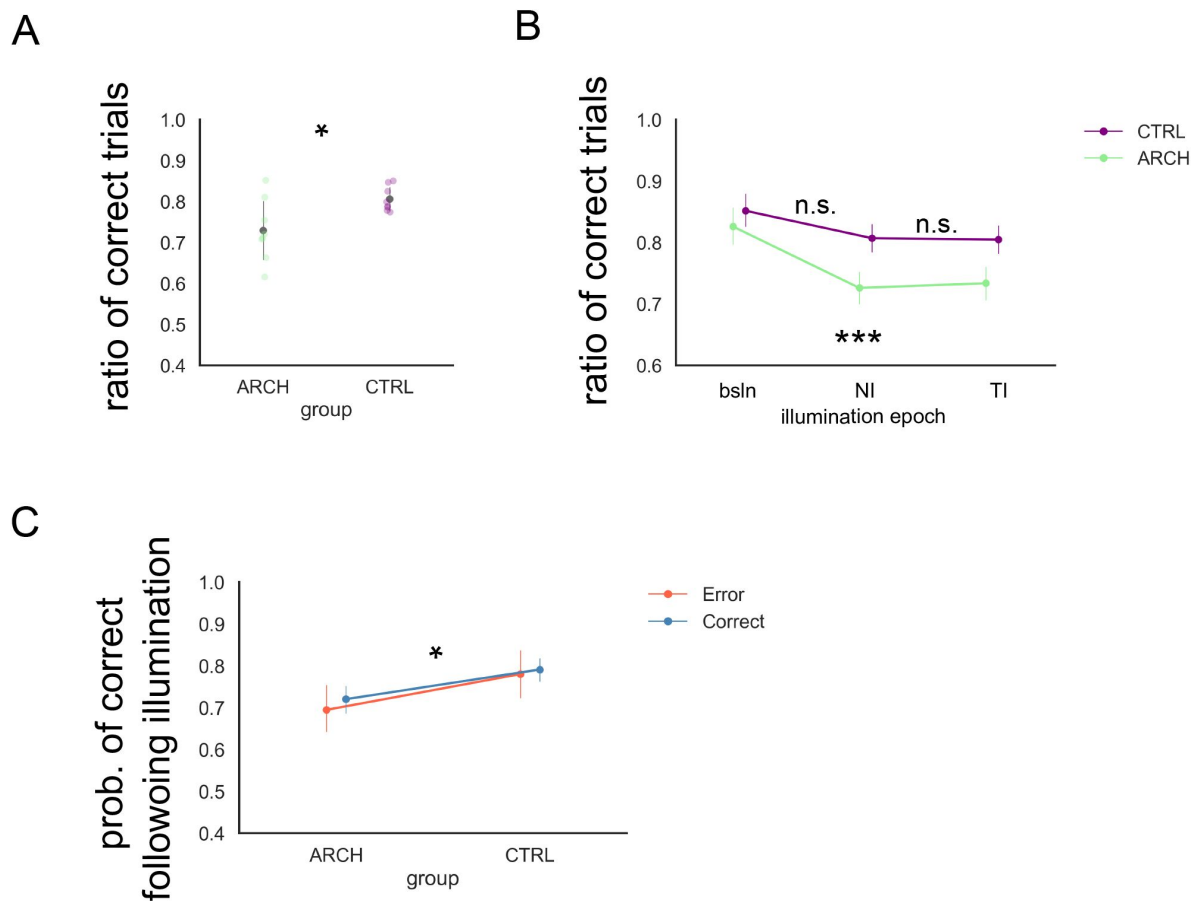


Figure 3.

Optogenetic silencing of HIPP terminals in RSC causes a significant and prolonged decrease in performance

(A) Illumination of RSC during DNMP leads to a significantly lower performance in eArch+, compared to empty-vector CTRL animals (CTRL: 0.81 ± 0.03 , ARCH: 0.73 ± 0.08 , GLMM, $p=0.006$, Bonferroni *post-hoc* test). (B) In eArchT+ animals, we found no differences between performance in TI and NI trials taken as groups, both significantly lower than both baseline ($p<0.001$), and the corresponding trial groups in CTRL animals (TI, $p=0.02$, NI, $p=0.008$). In CTRL animals no differences were found among the three groups ($p=0.252$ and 0.184) (C) Illumination on any given trial resulted in lower performance in the subsequent trial in eArch+, but not CTRL, animals ($p=0.015$), regardless of whether the illuminated trial is a correct or an incorrect trial. All summary data depicted is mean $\pm$ SD.

errors. To test such prediction, we analyzed the probability of an error trial following error, in illuminated sessions, and found that it is significantly higher in eArch+ animals than in CTRL animals, regardless of whether the present trial is itself illuminated (**Figure 4A** [↗](#), Table 5, $p < 0.001$, Bonferroni *post-hoc* test). Importantly, this difference is absent in baseline sessions, in which no illumination occurs (**Figure 4B** [↗](#), Table 6, $p = 0.172$, Bonferroni *post-hoc* test). This is consistent with our prediction, and suggests that silencing HIPP terminals in RSC interrupts the flow of information from the previous trial, preventing subsequent error-correction for which it is necessary.

How long does the effect of eArch silencing last?

The silencing effects of light-mediated eArch activation on hippocampal axon terminals onto RSC neurons outlasted illumination, affecting subsequent trials, and lowering spatial working memory performance. We thus sought to determine how long would such an effect last. According to existing literature, once expressed in CA3-CA1 synaptic terminals, illumination of Arch with green-yellow light results in its activation into a powerful outward-moving proton pump, effectively hyperpolarizing neurons, independent of local membrane voltage (Krol et al., 2019 [↗](#); Miao et al., 2015 [↗](#)). Besides this effect, eArch was recently found to reduce the amplitude and slope of fEPSP from right after the onset to over 2 minutes after illumination offset, via a pH increase that is independent of hyperpolarization, and to cause a sustained decrease in synaptic vesicle release (El-Gaby et al., 2016 [↗](#)). Such sustained alkalinization, specific of synaptic boutons, lasts for several minutes post-activation of eArch, and has been replicated on striatal-cortical synapses as well (Mahn et al., 2016 [↗](#)). This previous data reporting the effects of eArch activation led us to further investigate, in our experimental conditions, how far in time would eArch behavioral effects last. We thus asked the following question: once one trial is illuminated, how many subsequent trials will be affected? To answer this question, we computed the probability of a correct trial on trial $t+1$, $t+2$, $t+3$, and $t+4$, following every illuminated trial. Our previous data shows that, overall, non-illuminated trials suffer the effects of illumination, resulting in more errors. However, by selecting the second previous and third previous trial as a trigger for our analysis, we will be biasing our analysis to include a few sequences of one or two interleaved non-illuminated trials, thus possibly attenuating the silencing effect. As depicted in **Figure 5** [↗](#) (see also Table 7), the probability of a trial being correct is lower in eArch+, compared to CTRL animals, whenever TI happens in the previous (**Figure 5A** [↗](#) T-1, $p = 0.01$, Bonferroni *post-hoc* test), the second previous (T-2, $p = 0.033$), and the third previous trial (T-3, $p = 0.036$), such difference becoming non-significant on condition T-4, when the fourth previous trial was illuminated (T-4, $p = 0.078$). Considering that the ITI is circa 20 sec, we can reasonably estimate that some non-illuminated trials occurred between this and the n -previous illuminated one, and that, despite this, the effects of eArch activation are still present, hence after ~ 3 min. To try and analyze these effects in more detail, without decreasing the sample size to non-interpretable numbers, we quantified performance specifically following sequences of two consecutively non-illuminated trials. We found that after such sequences there is still a significant impairment in eArch+ animals, in agreement with the previous analysis and with the notion that the effects of eArch silencing outlast its activation by light (**Figure 5B** [↗](#), $p = 0.023$, Mann-Whitney U).

Silencing HIPP terminals in RSC hastens decision processing at the Choice Point

In previous work we have examined the role CG neurons might play during spatial WM, by silencing CG and measuring performance in this same DNMP task, and found that silencing CG interferes with performance, with animals spending less time in the decision processes in DNMP trials (Cruz et al., 2023 [↗](#)). We interpreted this decreased latency as hastiness, with animals taking erroneous decisions based on incomplete cognitive processing. We asked the exact same question in the present work, and quantified latency to the choice point, and latency at the choice point,

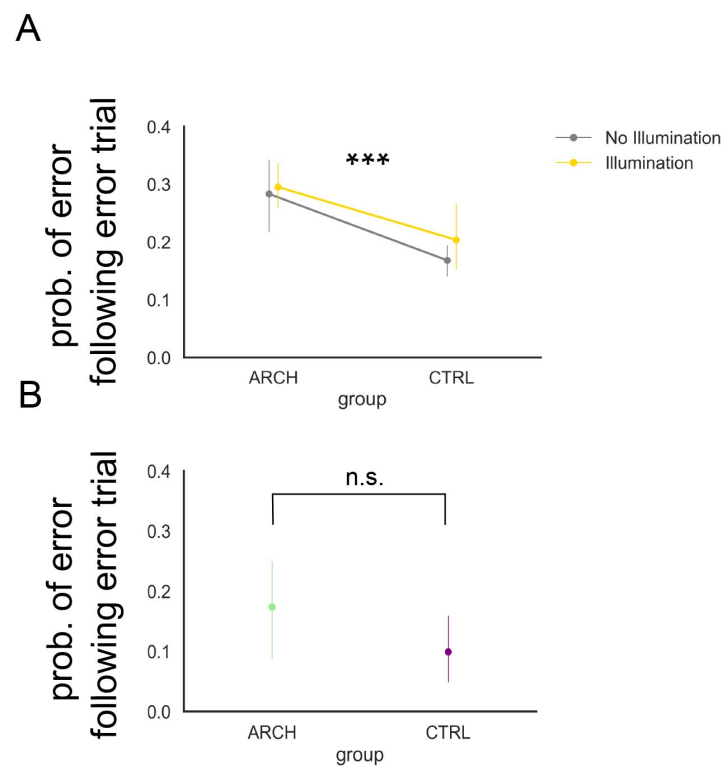


Figure 4.

Optogenetic silencing of HIPP terminals in RSC causes persistent error increases.

(A) eArch+ animals exhibit persistent errors ($p < 0.001$, Bonferroni *post-hoc* test). (B) Persistent errors are absent in baseline (no light delivered) sessions ($p = 0.172$, Bonferroni *post-hoc* test). All summary data depicted is mean $\pm$ SD.

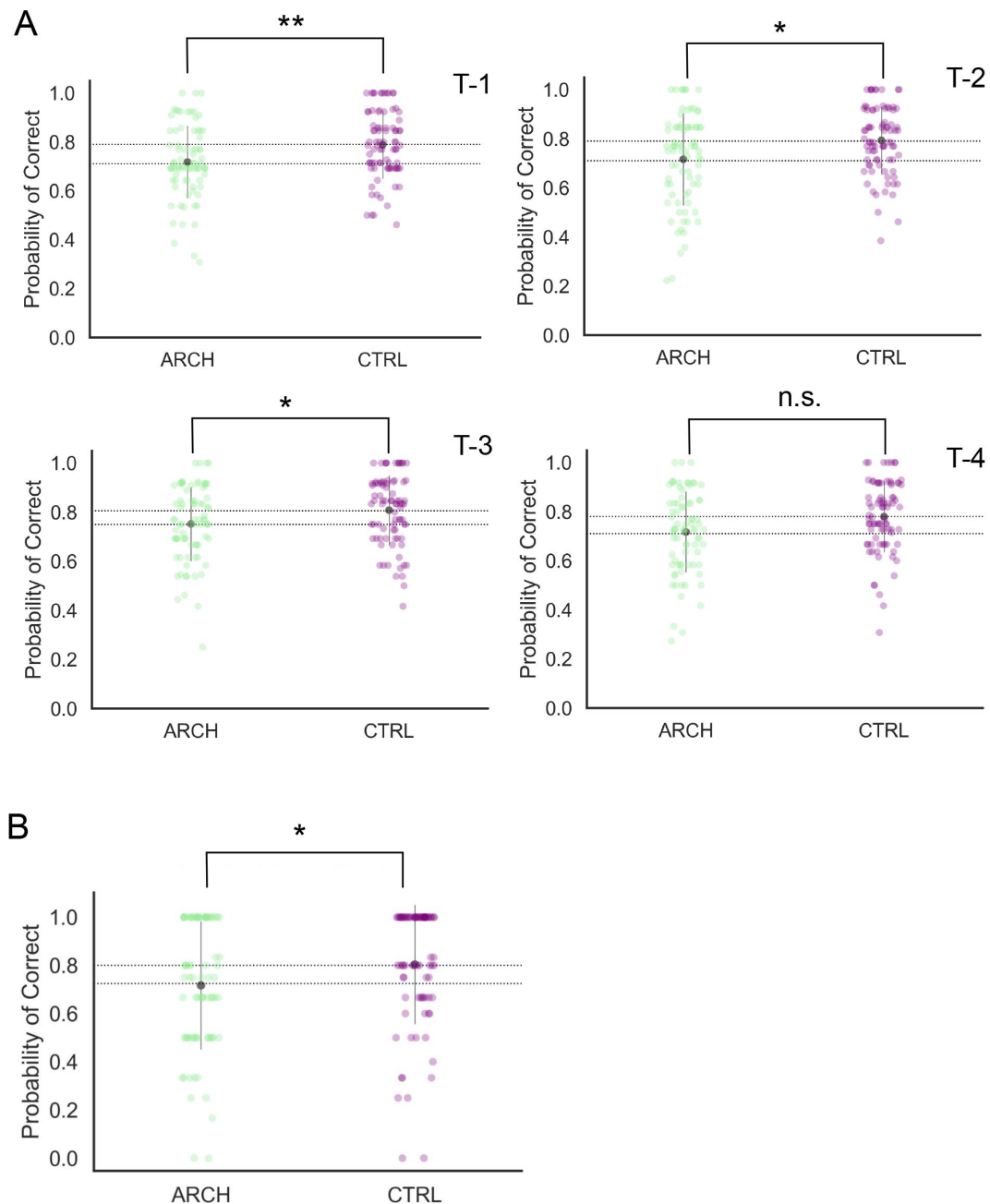


Figure 5.

Behavioral impairments resulting from optogenetic silencing of HIPP terminals in RSC outlast illumination

(A) eArch+ animals exhibit decreased performance once silencing occurred at T-1 ($p=0.01$, Bonferroni *post-hoc* test), T-2 ($p=0.033$), T-3 ($p=0.036$), not T-4 ($p=0.078$). (B) Two consecutively non-illuminated trials after illumination still result in decreased performance on the subsequent trial ($p=0.023$, Mann-Whitney U). All summary data depicted is mean $\pm$ SD.

overall, in error vs correct trials (**Figure 6**). We found that, overall, CTRL animals spend more time at the choice point than eArch+ animals (**Figure 6A**, Table 8, $p=0.02$, Fixed Effect Omnibus Test). Furthermore, this is true for Correct but not Error trials both under NI and TI (**Figure 6B**, respectively $p=0.008$ and $p=0.045$, Simple Effects Parameter Estimates), suggesting that in CTRL animals correct decisions involve more time taken at the choice point, contrary to error trials in which no such difference is found.

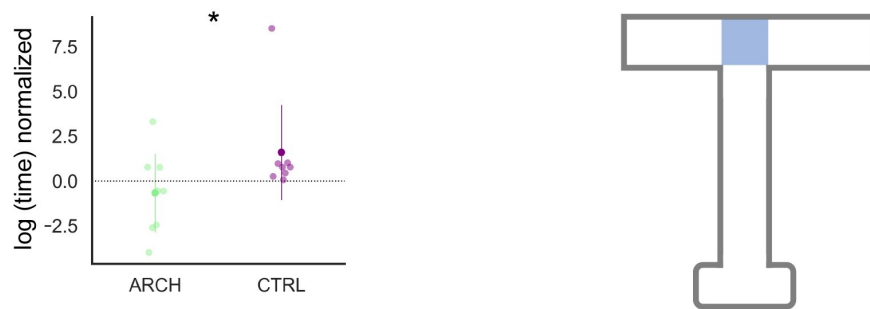
Discussion

We expressed eArch3.0 in hippocampal neurons and delivered green light into RSC, a cortical region thought to be involved in the encoding of contextual information relevant to represent future goals and actions, to silence HIPP terminals therein while rats ran a well-known spatial WM task. We found that such manipulation significantly decreased spatial working memory performance. Interestingly, we found that the effect of hippocampal terminal silencing in RSC during Test trials affects interleaved trials during which no light is delivered, thus spreading beyond illumination. We explored this effect further and found that eArch activation lowers performance down to the subsequent 3 non-illuminated trials, but also that it decreases error-corrective behavior, in which animals use action-outcome information after an error trial (i.e., absence of reward), to correct course in the subsequent trial. Such prolonged effect finds a precedent in a previous study (Robinson et al., 2017) in which silencing entorhinal-hippocampus circuits during WM trials results in lower performance in subsequent non-illuminated trials, suggesting that a possible short to mid-term neural plasticity event disrupts a key process at play during retrieval. Such an event might be related to recent findings showing that eArch activation in CA3-CA1 synaptic responses *in vitro* hyperpolarizes neurons independent of local membrane voltage (Krol et al., 2019; Miao et al., 2015), reducing the amplitude and slope of recorded fEPSP for over 2 minutes after illumination offset. This effect is achieved by a pH increase that causes a sustained decrease in synaptic efficacy, with lower vesicle release (El-Gaby et al., 2016) at synaptic boutons, replicated on another study using striatal-cortical synapses (Mahn et al., 2016). We believe such might be the mechanism behind the above-mentioned synaptic plasticity event. These findings imply that the temporal resolution of optogenetic neuron silencing, with neurons effectively unable of generating action potentials, cannot be generalized to the distinct realm of synaptic transmission. Rather, the effects of optogenetics in synaptic physiology might permeate into the synaptic plasticity mechanisms underlying memory acquisition and retrieval.

Finally, as we have seen before with optogenetic silencing of CG neurons during DNMP (Cruz et al., 2023), we found that eArch+ animals spend less time at the choice point compared to CTRL. This behavior is in line with hastier, more erroneous, decisions. In CTRL animals specifically, correct trials are characterized by an increased delay at the choice point, as if the correct decision process took place here. This is consistent with previous findings placing the behavioral decision at an equivalent stage in a continuous alternation task, sensitive to RSC permanent lesions, where goal locations are progressively encoded by RSC (Miller et al., 2019).

In sum, by reversibly silencing the HIPP synaptic inputs to RSC we have shown that silencing this input affects spatial working memory, possibly via changes in synaptic mechanisms conveying memorized contextual and episodic information about past decisions, and action-outcome associations, onto medial mesocortical executive decision centers.

A



B

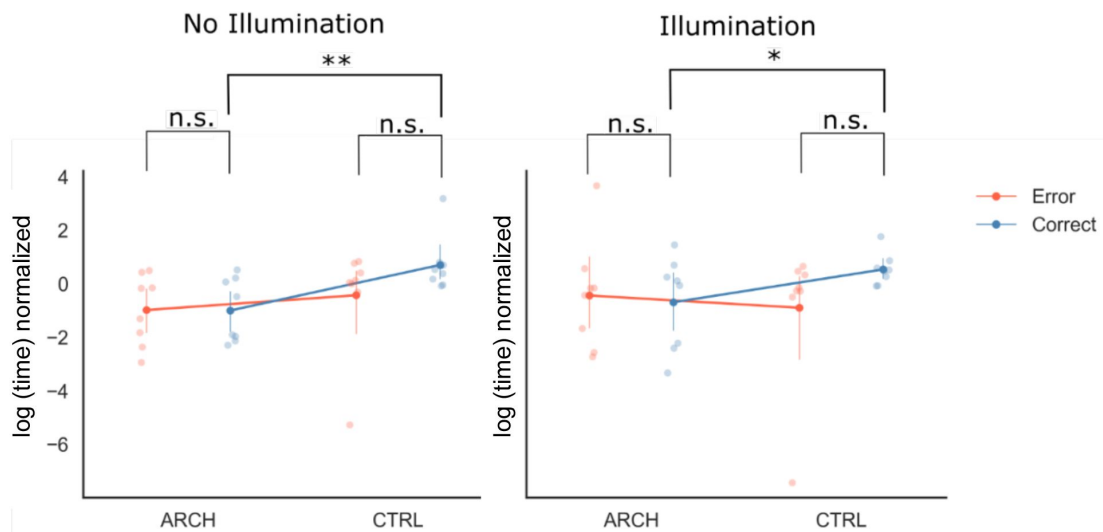


Figure 6.

Animals spend more time at the choice point in Correct trials unless HIPP terminals in RSC are silenced

(A) CTRL animals spend more time at the choice point than eArch+ animals ($p=0.02$, Fixed Effect Omnibus Test). (B) This is true specifically of Correct trials both in NI and TI (respectively $p=0.008$ and $p=0.045$, Simple Effects Parameter Estimates). All summary data depicted is mean $\pm$ SD.

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Author contributions

B P-C and MR designed the experiments, B P-C and PC-B conducted the experiments and analyzed the data, and MR wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

Lead contact and materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Miguel Remondes, DVM, PhD (mremondes@medicina.ulisboa.pt). This study did not generate new unique reagents.

Methods

Rats

We used 16 (8 eArch and 8 CTRL) male Long-Evans rats aged 4-6 months and weighing 430-600g (Charles River Laboratories), housed individually and kept under a 12hr light/dark cycle, gradually deprived to 80-85% of their free-feeding body weight. All procedures were performed in accordance with EU guidelines, approved by the Portuguese National Authority for Animal Health, as well as by the animal well-being office at Instituto de Medicina Molecular João Lobo Antunes.

Behavioral apparatus and equipment

Experiments were performed on a black acrylic T-maze comprising a central 170 cm long arm, a 25×30cm start platform, and a horizontal arm (88cm long) extending equally on both sides with two reward ports at their ends (2 cm diameter) where chocolate milk reward was delivered. A black plastic resting box (57215 cl:31139215 cl:31142 cm) was located near the start region to place the rats during the delay and inter-trial interval (ITI) periods. A cable tray above the maze supported the video recording and optogenetic stimulation systems. Both were linked to a computer situated in an adjacent room outside the behavior room. The Bonsai software (Lopes et al., 2015 [↗](#)) was used to process and control video recording, position tracking, reward delivery, and optogenetic stimulation onset.

To record the behavioral sessions, a Flea 3 PointGrey™ camera was used, capturing at a rate of 30fps. The optogenetic stimulation system used was the PlexBright system (Plexon, Dallas, TX, USA), comprising several components. The system consisted of an LD-1 Single Channel LED Driver set at 240 mA, connected to a Dual LED Commutator placed above the maze. This commutator was linked to two green 525nm LED modules, each attached to a 2-meter-long 0.66 NA patch cable with a FC ferrule tip and stainless-steel cladding. This patch tip was connected to an implanted lambda fiberoptic stub (200/230μm, 0.66 NA) with a 2mm active length and 5 mm implant length (Optogenix™) via a ceramic sleeve. Additionally, a red 5 mm LED was used to track the rats' xy position coordinates. This LED was connected to the tip of the patch cable, close to the fiberoptic stub implants.

Surgical procedure: viral injections and fiberoptic stub implants

Animals were bilaterally injected with the viral vector AAV5.hSyn-eArch3.0-eYFP and the respective control AAV5.hSyn-eYFP (University of North Carolina at Chapel Hill Vector Core). Viral titers were 4.4×10^{12} vg/ml and 3.3×10^{12} vg/ml, respectively. Before surgery, animals were placed in an induction chamber saturated with 5% isoflurane until absence of the righting reflex. Once the animals were deeply anesthetized, they were weighed and transferred to a heating pad. An intraperitoneal injection of a ketamine (37.5mg/kg) and medetomidine (0.5 mg/kg) cocktail (½ dose) was administered, as well as a subcutaneous injection of carprofen (5mg/kg, ½ dose), and Ringer's lactate every hour to maintain hydration levels. The heads were shaved, lidocaine applied to the ears, and eyes covered with a protection gel (LubriThal) to prevent drying of the cornea. The animals were installed in the stereotaxic frame secured by ear bars. Anesthesia was maintained with 1–3% isoflurane in oxygen until the end of the procedure. Once animals were placed on the frame, the surgical site was scrubbed with 10% povidone iodine alternated with 70% ethanol three times before an incision was made to expose the skull. Bregma and lambda were carefully identified and marked on the skull surface. These two landmarks were used to level the skull dorsoventrally. Once the skull was leveled, the craniotomy coordinates were marked on the bone. Before drilling, the bone surface was treated with Baytril to minimize infection.

A high-power microdrill tool was used to drill the location of four anchoring screws, on the lateral edges of the parietal bones, and to perform three craniotomies: two single-hole craniotomies (–5, ±4), one on each hemisphere, for the microinjection spots; and one craniotomy for the fiberoptic stubs covering both hemispheres. The latter was drilled in oval shape covering the fiberoptic stub implantation coordinates (–3, +1), (–5, –1). Loose bone pieces were carefully removed with tweezers. To prevent the brain from swelling while the next steps were performed, the craniotomies were covered with hemostatic sponge previously soaked in saline solution.

The viral construct was injected using a Narishige© manual microinjection control system operating a glass micropipette plunger, and both held on the stereotaxic frame. The micropipette was filled with mineral oil and placed in the microinjector, after which the viral suspension was aspirated (0.8μl). Using bregma as a reference, the pipette was carefully positioned over the microinjection coordinates and slowly lowered until reaching the targets: –5, ±4, –2.6 and –5, ±4, –2.8, where 0.4μl of viral suspension was injected after a 5 min resting period, at a rate of 100 nl/min. Once each injection was done, the micropipette was left in place for 10min before being slowly retracted. The craniotomies were then covered with hemostatic sponge previously soaked in saline solution and left in place to serve as a barrier between the brain and the dental acrylic (Kerr© TAB 2000 fast setting) applied afterwards.

In the fiberoptic stub craniotomy the dura was removed to expose the brain surface over each implant coordinate (–3, +1)(–5, –1). The fiberoptic stubs were secured to the stereotaxic frame, positioned in a 30° angle, and lowered across the two hemispheres to their final target position (–3, +1, –2.86)(–5, –1, –2.86). Following placement of the first fiberoptic stub it was secured using

dental acrylic cement (Kerr TAB 2000 fast setting) connecting it to the anchoring screws, while the opposite hemisphere was protected with hemostatic sponge. The same procedure was followed for the second fiberoptic stub.

Once the dental acrylic was cured, the skin was sutured using absorbable sutures, and 10% povidone iodine solution was applied around the wound. Carprofen (5mg/kg, $\frac{1}{2}$ dose) was administered subcutaneously for analgesia. Animals were then placed in a heated cage during the first 24h post-surgery for recovery. During this period, ad libitum food, nutritional gel, and water were provided. For the first 7 days, buprenorphine (0.3 mg/kg), a postoperative analgesic, was administered dissolved in chocolate milk.

Delayed non-matching to place task and protocol

During the week before surgery, rats underwent daily handling and were administered chocolate milk via a 1ml syringe for habituation. A week post-surgery, the process was repeated, and rats were habituated to the maze. To facilitate this habituation, chocolate milk drops were scattered across the maze and in the reward ports. Once the rats were drinking chocolate milk from the reward ports, they were habituated to do the same with the patch cords attached. Following habituation, the DNMP training sessions started.

Each session consisted of 28 trials, and each trial comprises three epochs: (1) the sample run, (2) the delay, (3) and the test run. (1) In the Sample Run, one arm was blocked, thereby the rat was forced to visit the only available arm, where a reward awaited; (2) when the rat consumed the reward, it was picked up and placed in the resting box for 15s. During this delay, the arms and the stem near the choice point were cleaned with 70% ethanol; (3) in the Test Run the block from epoch 1 was removed, rats were free to choose which arm to visit (left or right), and were rewarded for choosing the arm opposite to the Sample arm (i.e., non-matching the sample). After the Test Run, rats were returned to the box for a 20s ITI and a new trial started.

The Sample Run arms were balanced (50% left and 50% right), and pseudo-randomized to avoid more than 3 consecutive runs to the same arm. Rats were scored based on the number of correct and incorrect choices in the Test Run. Throughout the sessions, the position of the experimenter in the room remained constant. Rats were trained until reaching a performance of at least 75% of correct choices in 3 consecutive sessions, after which we introduced optogenetic inhibition.

Optogenetic inhibition protocol

The protocol consisted in 10 consecutive sessions of 28 trials, of which 14 were illuminated in the Test epoch (TI) and 14 were not illuminated (NI). This interleaved approach was chosen to prevent compensatory mechanisms. The illuminated trials had the arms pseudo-randomized, ensuring a balanced distribution of left and right sample arms (50% each), and restricted to allow a maximum of 3 consecutive trials. The onset of illumination was triggered to the position of the animal at 120 cm into the central arm and ended when the rat reached the chosen arm, at 190 cm. Based on the average speed of the animal at this point ($\sim 50\text{cm}\cdot\text{s}^{-1}$), we estimate illumination time to be ~ 1.4 sec. Each rat completed a total of 280 trials, half of which were subjected to optogenetic inhibition. Upon concluding the experiment, the rats were sacrificed, the implant was extracted, and the fiberoptic stubs were connected to the optogenetic system to verify the emission of light through the optic fiber.

Histology

At the end of the experiments, animals were sacrificed via an isoflurane overdose followed by an intraperitoneal injection of sodium pentobarbital (800 mg/kg). Rats were then transcardially perfused with 250 ml of PBS followed by 500 ml of 10% formalin solution. The brains were dissected and kept in a falcon tube containing 10% formalin solution, covered with aluminum foil,

and left at room temperature for 24h. Afterwards, the brains were transferred to a 15% sucrose solution and kept at 4°C overnight. Once sunk, the brains were embedded in gelatin, frozen in 2-Methylbutane liquid nitrogen, and sectioned in 50µm coronal slices using a cryostat (model CM3050 S, Leica). The fixed brain slices were degelatinized and incubated in DAPI 1:700 (Sigma) for 20 min. These slices were then mounted, cover slipped in Mowiol, and left to dry for 24h. The expression of the eArch3.0 viral construct in the hippocampus was confirmed using an AxioZoom V16 fluorescence stereo microscope (Zeiss). The expression of the eArch3.0 viral construct in the RSC was confirmed using a LSM 880 confocal point-scanning microscope with Airyscan (Zeiss). CTRL animals underwent the exact same procedure.

Data and statistical analyses

Datasets were manually assembled containing specific details of each run. Within this dataset, each session's runs were detailed with the following specifications: run number, run type (sample or test), illumination condition (TI or NI), and outcome (correct or incorrect trial). Additionally, the rats' xy positions were tracked via a LED, recorded, and timestamped using Bonsai software (Lopes et al., 2015 [DOI](#)). Custom Python scripts were employed to compute individual runs from these data, ensuring the accuracy of individual runs while excluding any with position data interference. Performance was computed for each experimental session including baseline sessions, consisting in the three sessions before starting optogenetic inhibition. Performance for illuminated and non-illuminated conditions was calculated as the ratio of correct trials over the total number of illuminated or non-illuminated trials, respectively. Probability computations were conducted for trial accuracy given the previous trial's illumination (trial_{n-1} to trial_{n-4}) and for error occurrence given the previous error trial, as follows:

- $(\text{correct trial}_n \mid \text{trial}_{n-x} \text{ illuminated}) = P(\text{trial}_n \text{ is correct} \cap \text{trial}_{n-x} \text{ is illuminated}) / P(\text{illuminated trials}_{n-x})$

$P(\text{error trial}_n \mid \text{trial}_{n-1} \text{ error}) = P(\text{trial}_n \text{ is an error} \cap \text{trial}_{n-1} \text{ is an error}) / P(\text{error trials}_{n-1})$ The time spent at the choice point was determined as the difference between the timestamps of choice point entry and exit. An additional 10cm was added to the region of interest to account for instances when the rat was stationary but engaged in head poking. Time values were Log-transformed to achieve a Gaussian distribution. Normalization to the baseline of log-transformed time in the choice point to the baseline was computed as: $[\log(\text{latency}) - \log(\text{baseline mean})] / [\log(\text{baseline mean})]$. Outliers in time data were identified as values deviating by 2 standard deviations (SD) from the mean and were subsequently removed.

Generalized Linear Mixed Models (GLMM) were employed to analyze the results. Model selection was based on the Akaike Information Criterion (AIC), with the chosen model being the one that converged and exhibited the lowest AIC value. The normality of a distribution was assessed using the Shapiro test along with assessment of residuals using Quantile-Quantile (Q-Q) plots. All the analysis was conducted in Jamovi with the GAMLJ package (Jonathon Love, 2022).

To assess baseline performance differences between eArch and CTRL groups, we used a GLMM with a binomial distribution and logit link function, with individual subjects (i.e., rats) as the random effects variable. The same GLMM, followed by a Bonferroni post-hoc test, was conducted to analyze the ratio of correct trials between groups during the experiment, and for assessing the trial history effect of illumination. The same tests were used to analyze the trial history effect of errors, using as random effect variables the “session number” and “run number”. To compare the groups after two consecutive illuminated trials, a Mann-Whitney U test was applied. For analyzing Log time in the choice point, a GLMM with a Gaussian distribution and identity link function was applied, considering rat as the random effects variable. To determine significant differences between groups we used the Wald Chi-Squared test, and for differences between groups and illumination condition we used simple effects parameter estimates.

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Editors

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Reviewer #2 (Public Review):

The authors examine the impact of optogenetic inhibition of hippocampal axon terminals in the retrosplenial cortex (RSP) during the performance of a working memory T-maze task. Performance on a delayed non-match-to-place task was impaired by such inhibition. The authors also report that inhibition is associated with faster decision-making and that the effects of inhibition can be observed over several subsequent trials. The work seems reasonably well done and the role of hippocampal projections to retrosplenial cortex in memory and decision-making is very relevant to multiple fields. However, the work should be expanded in several ways before one can make firm conclusions on the role of this projection in memory and behavior.

Comments on revised version:

The authors have provided their comments on the concerns voiced in my first review. I remain of the opinion that the experiments do not extend beyond determining whether disruption of hippocampal to retrosplenial cortex connections impacts spatial working memory. Given the restricted level of inquiry and the very moderate effect of the manipulation on memory, the work, in my opinion, does not provide significant insight into the processes of spatial working memory nor the function of the hippocampal to retrosplenial cortex connection.

<https://doi.org/10.7554/eLife.96515.2.sa0>

Author response:

The following is the authors' response to the original reviews.

eLife assessment

The authors report that optogenetic inhibition of hippocampal axon terminals in retrosplenial cortex impairs the performance of a delayed non-match to place task. The significance of findings elucidating the role of hippocampal projections to the retrosplenial cortex in memory and decision-making behaviors is important. However, the strength of evidence for the paper's claims is currently incomplete.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

This is a study on the role of the retrosplenial cortex (RSC) and the hippocampus in working memory. Working memory is a critical cognitive function that allows temporary retention of information for task execution. The RSC, which is functionally and anatomically connected to both primary sensory (especially visual) and higher cognitive areas, plays a key role in integrating spatial-temporal context and in goal-directed behaviors. However, the specific contributions of the RSC and the hippocampus in working memory-guided behaviors are not fully understood due to a lack of studies that experimentally disrupt the connection between these two regions during such behaviors.

In this study, researchers employed eArch3.0 to silence hippocampal axon terminals in the RSC, aiming to explore the roles of these brain regions in working memory. Experiments were conducted where animals with silenced hippocampal axon terminals in the RSC performed a delayed non-match to place (DNMP) task. The results indicated that this manipulation impaired memory retrieval, leading to decreased performance and quicker decision-making in the animals. Notably, the authors observed that the effects of this impairment persisted beyond the light-activation period of the opsin, affecting up to three subsequent trials. They suggest that disrupting the hippocampal-RSC connection has a significant and lasting impact on working memory performance.

Strengths:

They conducted a study exploring the impact of direct hippocampal inputs into the RSC, a region involved in encoding spatial-temporal context and transferring contextual information, on spatial working memory tasks. Utilizing eArch3.0 expressed in hippocampal neurons via the viral vector AAV5-hSyn1-eArch3.0, they aimed to bilaterally silence hippocampal terminals located at the RSC in rats pre-trained in a DNMP task. They discovered that silencing hippocampal terminals in the RSC significantly decreased working memory performance in eArch+ animals, especially during task interleaving sessions (TI) that alternated between trials with and without light delivery. This effect persisted even in non-illuminated trials, indicating a lasting impact beyond the periods of direct manipulation. Additionally, they observed a decreased likelihood of correct responses following TI trials and an increased error rate in eArch+ animals, even after incorrect responses, suggesting an impairment in error-corrective behavior. This contrasted with baseline sessions where no light was delivered, and both eArch+ and control animals showed low error rates.

Weaknesses:

While I agree with the authors that the role of hippocampal inputs to the RSC in spatial working memory is understudied and merits further investigation, I find that the optogenetic experiment, a core part of this manuscript that includes viral injections, could be improved. The effects were rather subtle, rendering some of the results barely significant and possibly too weak to support major conclusions.

We thank Reviewer#1 for carefully and critically reading our manuscript, and for the valuable comments provided. The judged “subtlety” of the effects stems from a perspective according to which a quantitatively lower effect bears less biological significance for cognition. We disagree with this perspective and find it rather reductive for several reasons.

Once seen in the context of the animal’s ecology, subtle impairments can be life-threatening precisely because of their subtlety, leading the animal to confidently rely on a defective capacity, for such events as remembering the habitual location of a predator, or food source.

Also, studies in animal cognition often undertake complete, rather than graded, suppression of a given mechanism (in the same sense as that of “knocking out” a gene that is relevant for behaviour), leading to a gravelly, rather than gradually, impaired model system, to the point of not allowing a hypothetical causal link to be mechanistically revealed beyond its mere presence. This often hinders a thorough interpretation of the perturbed factor’s role. If a caricatural analogy is allowed, it would be as if we were to study the role of an animal’s legs by chopping them both off and observing the resulting behaviour.

In our study we conclude that silencing HIPP inputs in RSC perturbs cognition enough to impair behaviour while not disabling the animal entirely, as such allowing for behaviour to proceed, and for our observation of graded, decreased (not absent), proficiency under optogenetic silencing. So rather than weak, we would say the results are statistically significant, and biologically realistic.

Additionally, no mechanistic investigation was conducted beyond referencing previous reports to interpret the core behavioral phenotypes.

We fully agree with this being a weakness, as we wish we could have done more mechanistic studies to find out exactly what is Arch activation doing to HIPP-RSC transmission, which neurons are being affected, and perhaps in the future dissect its circuit determinants. We have all these goals very present and hope we can address them soon.

Reviewer #2 (Public Review):

The authors examine the impact of optogenetic inhibition of hippocampal axon terminals in the retrosplenial cortex (RSP) during the performance of a working memory T-maze task. Performance on a delayed non-match-to-place task was impaired by such inhibition. The authors also report that inhibition is associated with faster decision-making and that the effects of inhibition can be observed over several subsequent trials. The work seems reasonably well done and the role of hippocampal projections to retrosplenial cortex in memory and decision-making is very relevant to multiple fields. However, the work should be expanded in several ways before one can make firm conclusions on the role of this projection in memory and behavior.

We thank Reviewer#2 for carefully and critically reading our manuscript, and for the valuable comments provided.

(1) The work is very singular in its message and the experimentation. Further, the impact of the inhibition on behaviour is very moderate. In this sense, the results do not support

the conclusion that the hippocampal projection to retrosplenial cortex is key to working memory in a navigational setting.

As we have mentioned in response to Reviewer#1, the judged “very moderate” effect stems from a perspective according to which a quantitatively lower effect bears less biological significance for cognition, precluding its consideration as “key” for behaviour. We disagree with this perspective and find it rather reductive for several reasons. Once seen in the context of the animal’s ecology, quantitatively lower impairments in working memory are no less key for this cognitive capacity, and can be life-threatening precisely because of their subtlety, leading the animal to confidently rely on a defective capacity, for such events as remembering the habitual location of a predator, or food source. Furthermore, studies in animal cognition often undertake complete, rather than graded, suppression of a given mechanism (in the same sense as “knocking out” a gene that is relevant for behaviour), leading to a gravelly, rather than gradually, impaired model system, to the point of not allowing a hypothetical causal link to be mechanistically revealed beyond its mere presence. This often hinders a thorough interpretation of its role.

In our study we conclude that silencing HIPP inputs in RSC perturbs behaviour enough to impair behaviour while not disabling the animal entirely, as such allowing for behaviour to proceed, and our observation of graded, decreased (not absent), proficiency under optogenetic silencing. So rather than weak, we would say the results are statistically significant, and biologically realistic.

(2) There are no experiments examining other types of behavior or working memory. Given that the animals used in the studies could be put through a large number of different tasks, this is surprising. There is no control navigational task. There is no working memory test that is non-spatial. Such results should be presented in order to put the main finding in context.

It is hard to gainsay this point. The more thorough and complete a behavioural characterization is, the more informative is the study, from every angle you look at it. While we agree that other forms of WM would be quite interesting in this context, we also cannot ignore the fact that DNMP is widely tested as a WM task, one that is biologically plausible, sensitive to perturbations of neural circuitry known to be at play therein, and fully accepted in the field. Faced with the impossibility of running further studies, for lack of additional funding and human resources, we chose to run this task.

A control navigational task would, in our understanding, be used to assess whether silencing HIPP projections to RSC would affect (spatial?) navigation, rather than WM, thus explaining the observed impairment. To this we have the following to say: Spatial Navigation is a very basic cognitive function, one that relies on body orientation relative to spatial context, on keeping an updated representation of such spatial context, (“alas”, as memory), and on guiding behaviour according to acquired knowledge about spatial context. Some of these functions are integral to spatial working memory, as such, they might indeed be affected.

Dissecting the determinants of spatial WM is indeed an ongoing effort, one that was not the intention of the current study, but also one that we have very present, in hope we can address in the future.

A non-spatial WM task would indeed vastly solidify our claims beyond spatial WM, onto WM. We have, for this reason, changed the title of the manuscript which now reads “spatial working memory”.

(3) The actual impact of the inhibition on activity in RSP is not provided. While this may not be strictly necessary, it is relevant that the hippocampal projection to RSP includes,

and is perhaps dominated by inhibitory inputs. I wonder why the authors chose to manipulate hippocampal inputs to RSP when the subiculum stands as a much stronger source of afferents to RSP and has been shown to exhibit spatial and directional tuning of activity. The points here are that we cannot be sure what the manipulation is really accomplishing in terms of inhibiting RSP activity (perhaps this explains the moderate impact on behavior) and that the effect of inhibiting hippocampal inputs is not an effective means by which to study how RSP is responsive to inputs that reflect environmental locations.

We fully agree that neural recordings addressing the effect of silencing on RSC neural activity is relevant. We do wish we could have provided more mechanistic studies, to find out exactly what is Arch activation doing to HIPP-RSC transmission, which neurons are being affected, and thus dissecting its circuit determinants. We have all these goals very present and hope we can address them soon. Subiculum, which we mention in the Introduction, is indeed a key player in this complex circuitry, one whose hypothetical influence is the subject of experimental studies which will certainly reveal many other key elements.

(4) The impact of inhibition on trials subsequent to the trial during which optical stimulation was actually supplied seems trivial. The authors themselves point to evidence that activation of the hyperpolarizing proton pump is rather long-lasting in its action. Further, each sample-test trial pairing is independent of the prior or subsequent trials. This finding is presented as a major finding of the work, but would normally be relegated to supplemental data as an expected outcome given the dynamics of the pump when activated.

We disagree that this finding is “trivial”, and object to the considerations of “normalcy”, which we are left wondering about.

In lack of neurophysiological experiments (for the reasons stated above) to address this interesting finding, we chose to interpret it in light of (the few) published observations, such being the logical course of action in scientific reporting, given the present circumstances.

Evidence for such a prolonged effect in the context of behaviour is scarce (to our knowledge only the one we cite in the manuscript). As such, it is highly relevant to report it, and give it the relevance we do in our manuscript, rather than “relegating it to supplementary data”, as the reviewer considers being “normal”.

In the DNMP task the consecutive sample-test pairs are explicitly not independent, as they are part of the same behavioural session. This is illustrated by the simple phenomenon of learning, namely the intra-session learning curves, and the well-known behavioral trial-history effects. The brain does not simply erase such information during the ITI.

(5) In the middle of the first paragraph of the discussion, the authors make reference to work showing RSP responses to "contextual information in egocentric and allocentric reference frames". The citations here are clearly deficient. How is the Nitzan 2020 paper at all relevant here?

Nitzan 2020 reports the propagation of information from HIPP to CTX via SUB and RSC, thus providing a conduit for mnemonic information between the two structures, alternative to the one we target, thus providing thorough information concerning the HIPP-RSC circuitry at play during behaviour.

Alexander and Nitz 2015 precisely cite the encoding, and conjunction, of two types of contextual information, internal (ego-) and external (allocentric).

The subsequent reference is indeed superfluous here.

We thank the Reviewer#2 for calling our attention to the fact that references for this information are inadequate and lacking. We have now cited (Gill et al., 2011; Miller et al., 2019; Vedder et al., 2017) and refer readers to the review (Alexander et al., 2023) for the purpose of illustrating the encoding of information in the two reference frames. In addition, we have substantially edited the Introduction and Discussion sections, and suppressed unnecessary passages.

(6) The manuscript is deficient in referencing and discussing data from the Smith laboratory that is similar. The discussion reads mainly like a repeat of the results section.

Please see above. We thank Reviewer#2 for this comment, we have now re-written the Discussion such that it is less of a summary of the Results and more focused on their implications and future directions.

Response to recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major

Line 101: Even with the tapered lambda fibre optic stub, if the fibre optics were longitudinally staggered by 2 millimetres, they would deliver light to diagonal regions in the horizontal plane rather than covering the full length of the RSC. Is this staggering pattern randomized or fixed? Additionally, Figure 1C is a bit misleading, as the light distribution pattern from the tapered fibre optic is likely to be more concentrated near the surface of the fibre, rather than spreading widely in a large spherical pattern.

The staggering is fixed. The elliptical (not spherical) contour in Fig 1C is not meant to convey any quantitative information, but rather to visually orient the reader towards the directions into which light will likely propagate, the effects of which we do not attempt to estimate here. We have made this contour smaller.

Line 119: The authors demonstrate the viral expression pattern of a representative animal and the overall expression patterns of all other animals in Figure 1 and the Supplementary Figures. However, numerous cases in the Supplementary Figures exhibit viral leakages and strong expressions in adjacent cortical and thalamic areas. Although there is a magnified view of the RSC's expression pattern in Figure 1, authors should show the same way in the supplemental data as well. Additionally, the degree of viral expression in the hippocampal subregions varies substantially across animals. This variation is concerning and impacts the interpretation of the results.

The viral construct was injected in the HIPPO at coordinates based on our previous work (Ferreira-Fernandes et al., 2019) wherein injections of a similar vector in mid-dorsal HIPPO resulted in widespread expression throughout the medial mesocortex AP extent, RSC through CG, as well as other areas in which HIPPO establishes synapses. These were studied in detail then, by estimating the density of axon terminals. In the present work we did not acquire high-mag images of all slices, since they were too expensive, and we had this information from the study above. Still, we have now added further examples of high-mag images taken from eArch and CTRL animals.

We believe it is important here to mention the fact that the virus we use, AAV5, only travels anterograde and is static (i.e. it does not travel transynaptically).

Variations in viral expression are to be expected even if injections happen in the exact same way. It is crucial then, that fibre positioning is constant across animals, to guarantee that its

relationship with viral expression is thence consistent, and to render irrelevant whatever off-target expression of the viral construct. We have ascertained this condition post-mortem in all our animals.

Line 124: Another point regarding the viral expressions and optical fibre implants used to inhibit the HIPPO-RSC pathway is that the RSC and HIPPO extend substantially along the anterior-posterior axis. The authors should demonstrate how the viral expression is distributed along this axis and indicate where the tip of the tapered optical fibre ended by marking it in the histological images. This information is crucial to confirm the authors' claim that the hippocampal projection terminals were indeed modulated by optical light. Also, the manuscript would benefit from details about the power/duration and/or modulation of the light used.

In both Figures 1 and S1 panels we can clearly see the tracks formed by the fibres. This provides examples of such dual angle placement vis a vis the expression of the construct, demonstrating that the former is fully targeted towards the latter. We have added markers to highlight these tracks and an example of a “full” track in figure S1. We did not have animals deviating from this relative positioning to any significant extent. The methods section mentions illumination power as 240mA, and we have now added estimated illumination time as well.

Line 141: The authors should include data on task performance during learning and baseline sessions for each animal, to demonstrate that they fully grasped the task rules and that achieving a 75% performance ratio was sufficient.

DNMP is a standard WM task used for many decades, in which animals reach performances above 75% in 4-8 sessions. We have used it extensively, and never saw any deviations from this learning rate and curve. We ran daily sessions until animals reached 75%, and thereafter until they maintained this performance, or above, for three consecutive sessions (the data points we show). We saw no deviations from what is published, nor from what is our own extensive experience, and thence are fully confident that all animals included in this manuscript grasped task rules.

Line 146: While the study focused on inhibiting inputs during the test run (retrieval phase), it would be beneficial to also inhibit inputs during the sample run (encoding phase) and the delay period. This would help confirm whether the silencing affects only working memory retrieval, or if it also impacts encoding and maintenance.

We agree, it would be very interesting to determine if there are any effects of silencing HIPPO RSC terminals during Sample. However, since there is a limit to the number of trials per session, and to the total number of sessions, we could not run the three manipulations within each session of our experimental design, as that would lower the number of trials per condition to an extent that would affect statistical power. Silencing HIPPO RSC terminals during Sample would best be a separate experiment, asking a different question, and perhaps within an experimental design distinct from the one envisioned.

A very important point here relates to the fact that the effects of optogenetic manipulation do not limit themselves to the illumination epoch, in fact they extend far beyond onto the 3rd trial post-illumination. The insertion of Sample-illuminated trials interleaved in the same session would fundamentally affect the interpretation of experimental results, as we could not attribute lower performances to the effects in either or both manipulated epochs.

Line 225: Figure 5 illustrates that silencing the inputs results in an extended impairment of working memory performance. However, it's unclear if there are any behavioural

changes during the sample run. The inhibition could potentially affect encoding in the subsequent sample run, considering the inter-trial interval (ITI) is only 20 seconds.

From the observation of behaviour and the analysis of our data, we saw no overt “behavioural changes during the sample run”, as latencies and speeds were essentially unchanged.

If what is meant by your comment is the effect of optogenetic manipulation being protracted from the Test towards the Sample epoch, we find this unlikely. Conservatively, we estimate the peak of our optogenetic manipulation to occur around the time light is delivered, the Test phase, rather than 20-30 secs later.

In theory, any effect of optogenetic silencing of HIPP terminals in RSC can cause disturbances in encoding or Sample, the ITI itself, and the epoch in which mnemonic information retrieved from the Sample epoch is confronted with the contextual information present during Test, leading to a decision. This is regardless of the illumination epoch, and even if the effect of optogenetic manipulation is not prolonged in time.

Since in our experiments we specifically target the Test epoch, and there is, in all likelihood, a decaying magnitude of neurophysiological effects, manifest in the reported decaying nature of the manipulation mechanism, and in our observed decrease of behavioural proficiency from subsequent trials 1:4, we are convinced that a conservative interpretation is that our major effect is concentrated in the epoch in which we deliver light - the Test epoch, the consequences of which (possibly related to short term plasticity events taking place within the HIPP-RSC neural circuit) extending further in time.

Line 410: The methods section on the surgical procedure could be clearer, particularly regarding the coordinates for microinjection and fibre implantation. A more precise description would aid reader comprehension.

The now-reported injection and implantation coordinates include the numbers corresponding to the distances, in mm, from Bregma to the targets, in the three stereotaxic dimensions considered: antero-posterior, medial-lateral left and right, and dorso-ventral, as well as the angle at which the fibres were positioned. We have added labels to the figures to highlight the fiberoptic track locations. We will be happy to provide further details as deemed necessary.

Line 461: It would be helpful to know if each animal displayed a preference for the left or right side. Including a description or figure showing that the performance ratio exceeded 75% in both left and right trials would provide a more comprehensive understanding of the animals' behaviour.

In the DNMP, an extensively used and documented WM task, it is an absolute pre-condition that no animals are biased to either side. As such, we did not use any animal that showed such bias.

We have not observed this to be the case in any of our candidate animals, nor would we use any animal exhibiting such a preference.

Minor

Line 25: In the INTRODUCTION section, the authors introduce ego-centric and allocentric variables in the RSC. However, if they intend to discuss this feature, there is no supporting data for ego-centric or allocentric variables in the Results section.

We agree. The extent of the discussion of ego vs allo-centric variables in our manuscript might venture a bit out of the main subject. It was included to provide wider context to our reporting of the data, considering that spatial working memory is indeed one instance in which egocentric- and allocentric-referenced cognitive mechanisms confront each other, and one in which silencing the HIPPO input to a cortical region thence involved would likely disturb ensuing computations. We have now substantially edited the manuscript's Introduction and Discussion, sections, namely toning down this aspect.

| *Line 125: In the section title, DNMT -> DNMP obviously.*

We have corrected this passage.

| *Figures: The quality of the figure panels does not meet the expected standards. For example, scale bars are missing in many panels (e.g., Figure 1A bottom, 1B, 1C, S1), figure labels are misaligned (as seen in Figure 3A-B compared to 3C, same with Figure 5), and there is inconsistency in color schemes (e.g., Figure 3C versus Figure 6, where 'Error' versus 'Correct' is depicted using green versus blue, respectively).*

We have now corrected these inconsistencies and mistakes.

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