nature portfolio

Peer Review File

The hippocampal CA3 area implements sequence learning of discontinuous episodes

Corresponding Author: Professor Suk-Ho Lee

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear Authors,

I have carefully reviewed the manuscript and commend the authors for tackling a challenging and important problem: how CA3 intrinsic excitability plasticity may support the linking of experiences into sequences. This is a creative and ambitious study that integrates slice physiology, computational modeling, and behavior. The work is intriguing, but in its current form the manuscript requires major revisions before it can be considered for publication. Below I provide a structured assessment.

Summary of the manuscript

The authors examine bi-directional regulation of intrinsic excitability (IE) in CA3 pyramidal neurons. Using c-fos–based viral tagging, they show that ensemble neurons activated in a contextual fear conditioning (CFC) paradigm display reduced input conductance and higher excitability. With repeated exposure, subgroups emerge: some maintain high excitability, others revert to baseline, interpreted as PP-driven reset. They link these effects to Kv1.2/D-type K^+ current reduction.

A computational CA3 model incorporates these rules to demonstrate ensemble linking without saturation. Finally, in a five-arm radial maze task, CA3-Kcna2+/– mice perform normally on simple cue-guided conditions but are impaired under high probe-load conditions (C1P3), which the authors interpret as a sequence learning deficit.

The manuscript aims to connect cellular ion channel mechanisms to ensemble linking and sequence behavior.

Conceptual issues and concerns about interpretation

Aversive conditioning confound: All physiology derives from CFC with foot shock, yet the Discussion treats exposures as neutral. Stress strongly affects CA3 excitability, and freezing behavior is not reported.

Synaptic vs intrinsic ambiguity: EPSP/EPSC changes could reflect synaptic AMPAR plasticity. AMPA/NMDA ratios were not measured, leaving open a purely synaptic explanation.

Model limitations: The network model uses 10% PC–PC connectivity (vs <2% in vivo) and artificially accelerated IE kinetics. It illustrates feasibility but does not provide predictive validation.

Behavioral interpretation: Only C1P3 genuinely requires sequence recall. C3P1 can be solved without true sequential memory. Analyses use only success/failure rates; no latency, trajectory, or trial-level mixed-effects analyses are provided. Motivation and stress controls are absent.

Writing clarity: Critical details are sometimes obscured, for example using “CFC” in figure legends without spelling out “contextual fear conditioning.” This makes it too easy to miss that aversive shock was administered.

Reporting gaps: Viral titers are not provided, infection-level controls are missing, and most recordings were under PTX.

Overall, while the dataset is rich, the claims currently overstep what the evidence directly supports.

Figure-by-figure evaluation

Figure 1 — Ensemble LTP-IE

Strength: clear demonstration of IE changes.

Concern: foot shock confound; recordings under PTX.

Needed: non-aversive controls, freezing data, intact inhibition recordings.

Figure 2 — GFP intensity

Strength: Alexa-594 normalization.

Concern: viral titer absent; GFP intensity confounded by infection load.

Needed: titer reporting, infection spread, and a constitutive GFP expression control to confirm GFP itself does not alter excitability.

Figure 3 — Double-activated subgroups

Strength: identification of heterogeneous IE responses.

Concern: clustering choices not justified. Importantly, exposure to context B (highly similar to A) could represent a recall session, not a new encoding event, complicating interpretation.

Needed: validation of clustering, explicit treatment of context B as recall.

Figure 4 — PP EPSP/EPSC

Strength: correlation with G_in.

Concern: lack of AMPA/NMDA ratios, PTX conditions.

Needed: AMPA/NMDA recordings, intact inhibition replications.

Figure 5 — D-type K^+ current

Strength: plausible link to IE.

Concern: 4-AP not Kv1.2-specific; no dendritic evidence.

Needed: α-DTX, normalization, kinetics, dendritic validation.

Figure 6 — Model encoding

Strength: conceptual linkage.

Concern: unrealistic connectivity and kinetics; no data validation.

Needed: sensitivity analyses, slower IE implementation.

Figure 7 — Model recall

Strength: illustrates sequence recall.

Concern: simplifications limit realism.

Needed: simulations with realistic IE timescales, predictions testable in physiology.

Figure 8 — Task design

Strength: clear schematics.

Concern: C3P1 does not test sequence memory; probe failures rescued with cues; only males; no motivation/anxiety controls.

Needed: sequence randomization, removal of within-run rescue, trajectory/latency analyses.

Figure 9 — Behavior results

Strength: WT vs mutants diverge under C1P3.

Concern: C3P1 not diagnostic; small/uneven Ns; binary data not analyzed with mixed-effects models.

Needed: logistic mixed-effects analysis, effect sizes, behavioral controls, and acute CA3 manipulations or Kv1.2 rescue to establish necessity.

Minor issues

Spell out “CFC” and provide freezing curves.

Report viral titers and infection spread.

Clarify animal-level Ns and apply hierarchical statistics.

Replicate key findings under intact inhibition.

State sex of animals consistently.

Model connectivity should reflect biological values or provide sensitivity testing.

Additional recommendations

The Discussion should include a Limitations and Weaknesses section, explicitly acknowledging the aversive conditioning context, possible synaptic contributions, illustrative modeling, and behavioral strategy alternatives.

Crucially, the study lacks causal manipulations:

A gain-of-function experiment (e.g., artificially boosting CA3 excitability plasticity and testing sequence behavior) would establish sufficiency.

A loss-of-function experiment (acute CA3 silencing or Kv1.2 rescue/knockdown during behavior) would test necessity. These are essential to establish a causal role for CA3 IE in sequence learning.

Conclusion and recommendation

This is an innovative and ambitious manuscript, but several conceptual, methodological, and interpretational issues need to be addressed. The physiology is intriguing, the modeling creative, and the behavioral design promising, yet the manuscript currently over-claims and omits critical controls.

Recommendation: Major revision. Addressing the aversive conditioning confound, adding synaptic assays, improving behavioral analysis, clarifying reporting, and including causal gain- and loss-of-function experiments will be necessary to substantiate the central claims.

In closing
This manuscript reflects substantial effort and creativity. With additional rigor, transparency, and acknowledgment of limitations, it has the potential to become a field-shaping contribution. I look forward to a strengthened revision.

Thanks,

Reviewer #2

(Remarks to the Author)
The authors of this manuscript use electrophysiology, modeling and behavioral approaches with the goal to understand how intrinsic plasticity determined by changes in IKD in CA3 neurons might contribute to sequence learning. The results might be potentially interesting; however I find that the manuscript is very difficult to read, especially in its first part, because of the way the findings are currently presented.
Some of the issues are detailed below.

1)The numbers for all the experimental (sub)groups presented should be included to be able to better evaluate the validity of the findings in function of the sample size.
2)It would be useful to have a summary figure to explain the framework, including the synaptic inputs and the kind of plasticity they undergo as they pertain to the various experiments, especially for researchers who are not deeply familiar with the specific topic.
3)The authors should clearly state their definition of CA3 ensemble cells in the beginning, as the term is mentioned but not

defined in the introduction (line 93). I assume that it means CA3 neurons that are activated in a specific context, but this in not clearly stated.
4)Some of the names of the experimental subgroups appear to be convoluted, making it difficult to follow the presentation of the findings (see for example ctxAB+, ctxAB++ as well ctxAB(++/1) and ctxAB(++/2) also mentioned below).
5)In Fig. 3, were ctxAB(++/1) and ctxAB(++/2) cells recorded from the same or different animals?

Minor:
Abstract lines 41-42: something is missing in this sentence.
Introduction, lines 59-63: reference should be added for these statements.
Results: is there a reason why the experiments in Fig. 1 are performed in rats whereas the rest are in mice?
Results, lines 145-148: it is unclear how these sentences refer to Fig. 2, since only fluorescence data is presented in this Figure.
Results, line 134: is ctxAB(++) here a typo? Because the two context experiments are not introduced until later.
Results, Figure 3Be: the term shEGFP(++) is not introduced anywhere in the manuscript.
Results, lines 236-238: the sentence is unclear.
Methods, line 538: the sentence about glutathione is unclear, since it is not mentioned elsewhere.

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors tested their hypothesis that Kv1.2 channels in CA3 pyramidal cells contribute to sequence learning. The group focuses on the role of Kv1.2 channels in cellular excitability for years. In this manuscript they expand their previous in vitro work to test their hypothesis in behaving mice and a computational model. They first demonstrate that exposure of mice to a novel context increases cellular excitability in a Kv1.1 and Kv1.2 channel dependent manner. Second, authors propose a computational model where this mechanism can form a sequential synaptic link. Third, they show that mice with knockdown of these channels are impaired in a sequence memory task. The combination of these results is novel and interesting, addressing the behavioral relevance to the potassium channel dependent plasticity of intrinsic excitability. These studies that aim to understand the role of molecular level mechanism in cognition have potentials to develop future treatments for cognitive deficits. However, there are several major issues as I describe below.

Major:
The behavioral task that the authors used does not support strongly the mechanism the authors are interested in is involved. This is because there is no temporal discontinuity in this task. Firing of place cells in CA3 that fire at different locations on the maze are known to overlap in time as the animals run. They can therefore be linked through a simple STDP mechanism without involving IKD mechanism that the authors focus on this paper. Better tasks are, for example, trace conditioning or sequence of odor presentations separated by temporal gaps, which have been shown to depend on hippocampus.

Grouping of cells and statistical analysis in ex vivo experiments are not scientifically appropriate in Fig1-4. In Fig1, to address the effect of cell activation, which is marked by the EGFP expression, Gin of all EGFP- and EGFP+ has to be compared. It is not scientifically correct to separate observed results into groups based on observed measurements (analysis is circular). The interpretation that “EGFP(-/low) cells may represent memory ensemble cells that are not infected by AAV” might be true. But it is not scientific to separate observed datapoints into groups based on such speculation. Authors need evidence.
Similarly, in Fig 3, the interpretation “Because cells with distinctly high G/R ratio were observed only in the mice which visited both context A and B, the ctxAB(++) cells may be activated in both contexts, while cells in the other group were activated once.” is a speculation. It is not correct to separate the results into two groups based on the value you observed from ctxAB group based on such speculation. It is obvious if one separates observed group into high and low data points that you will get significant difference between those groups (which is shown in Fig3 Be ctxAB+ vs ctxAB++ comparison with *** $p<0.0001$). Similarly, if you take the high value group of ctxAB group and compared to the ctxA+ group, finding out that ctxAB++ group is higher (which is shown in Fig3Be as well) is nothing to do with the fact that the animal went through ctx A and B, it could just be because authors took high values from ctxAB group.
In Fig3 C to E, it is necessary to have the ctxAB group which include all ctxAB+, ctxAB++1 and ctxAB++2 groups together. If further sub-categorization is needed, authors can add extra analysis where the authors focus on sub grouping of ctxAB group with careful justification.

In Fig2, how do we know that this difference of fluorescence intensity distribution is not simply due to the time from the last exposure to a context? To address this, context B only exposure followed by ex vivo experiment 60 min later is needed.

In the modeling study, authors used PP input to all groups (M1-M4). This is misleading considering the fact that the authors stress “orthogonal” memory representation. Since the model heavily rely on this, it should be justified based on empirical data.

Different mechanism that are proposed to support temporally separated events should be mentioned in the introduction and discussion (e.g. persistent activity, replay, short-term synaptic plasticity). In addition, other mechanisms involved in activity induced change in excitability should also be mentioned (e.g. H-current).

Minor:
It should be written in the abstract and introduction that labeling of active cells in context exposure was done in this study.

In the abstract, the statement that “Here we show that the high excitability of CA3 ensemble cells in the animals experienced an event can be normalized by a subsequent event.” is not supported. This, as the authors discuss in the Discussion, is an interpretation.
L 52: English needs to be checked by a native speaker. For example, “capture hypothesis may link two neuronal ensembles” is probably wrong because “hypothesis” does not “link” memories.
L 118: “Whole-cell patch“ -> “Whole-cell patch recording”?
L 134: ctxAB(++) appears first time here and it is not clear what it means and how this is relevant.
Fig1.B: It should be indicated from which group each EGFP- cells came from. Did the EGFP(-/low) group came from specific group (60, 90 or 180 min)?
Fig1.Cc: Is it a mistake that EGFP(-/high) shows n.s.?
Fig1.B: If the authors’ main claim is that inclusion of cells into assembly causes the reduction of Gin, then they should show statistical comparison of the 60 and 90 min group compared to control and EGFP- groups (without separating them into low and high).
L 216: underwent -> undergone
L 261: It helps if there is a summary statement of this section at the end.
Fig. 6 and 7: The text goes back and forth between Fig6 and 7. Modify the figures or the text to avoid back and forth.
L 344: The explanation of the task is hard to understand. Especially, it is not clear enough for the first time reader how the sequence is implemented. Instead of “A ‘run’ was completed, when a mouse visited four towers in a sequence (Fig. 8A).”, it would be better to write something like: “A sequence animal needed to learn was defined by a ‘run’ which consisted of four trials (i.e. visiting of four towers) “.
L 348: “guided run” should be defined.
L 390: This possibility that some ctxAB cells that went back to normal excitability level is a natural flow of the paper which is expected to be mentioned in the results section than the discussion section.
L417: “are lacking”
L423: “little has been understood”
L425: Logic around here is difficult to follow. Especially, it is often unclear if the word “overlap” is used for temporal overlap or spatial (neuronal representation) overlap . This is the case also in Line 36 “non-overlapping representation of memories”.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear authors,

The revised version of the manuscript has answered my concerns to a large extent. I have no further comments.

Congratulations on your interesting study.

Best Regards,

Reviewer #2

(Remarks to the Author)
The authors addressed most of the issues I raised in my prior review; however, there are still a couple of issues.

MAJOR
Figure 10 does not clarify and, if possible, makes the summary more confusing.
What I was asking was to identify which inputs (MF, vs PP vs A/C) are causing what type of plasticity and in which order. This is missing in the diagram. In addition, only event 1 and 2 of the four events in the diagram are described in the figure legend. Finally, at line 1039, does non-MF inputs refer to A/C input, PP inputs or both?

MINOR
Fig. 2 does not report the number of samples in this data set. In all other data sets, the number of samples is reported in the figure legends, except for Fig. 5 where they are reported in the text. This seems an odd choice; for consistency, it may be better to report them all in the figure legends.

Reviewer #3

(Remarks to the Author)
The first major concern I raised in the initial review remains: there is still a substantial gap between the behaviour task and the remaining. Increased excitability in the in vitro study was examined in relation to a novel context and aversive stimulation, neither of which is relevant in the 5-arm maze task. While the 5-arm maze task involves a sequence, the in vitro work used a novel context as the induction for IKD dependent mechanisms; therefore, it does not provide evidence that

increased excitability occurs when the mice visited each arm or when the light cue was turned on. My previous mention of trace fear conditioning was to potentially address this exact point. The authors have, however, indicated that trace fear conditioning was tested but was unaffected.
In the revision, the authors pointed out that the change in increased excitability was not mainly due to the footshock, but rather the new context. I find it difficult to reconcile the precisely timed input in the model (which induced increased excitability) with context exposure. As far as we currently know, context exposure lacks such precisely timed, distinct inputs. Such time-constrained stimuli are more consistent with a footshock. This, unfortunately, represents a new mismatch that I noticed in this revision.
The authors claimed that the 5-second temporal gap between trials provides a discontinuity that requires mechanisms longer-lasting than NMDA-dependent synaptic plasticity. However, studies indicate that place cells remain active during immobility (Hok et al, Journal of Neuroscience, 27 (3) 472-482, 2007). It has also been shown that “time cells” can bridge such temporal gaps of up to 5–10 seconds (Pastalkova et al., Science, 321(5894):1322-7, 2008). These indicates that hippocampal activity could bridge the 5-second gap. Therefore, the situation is unlike the simulation, where there is no input or firing in the hippocampus for 5 seconds. If this activity maintains the information about the current arm, the association between the current arm and the next light cue might be done with NMDA-dependent time scale. In fact, it has been reported that place cells and time cells can fire differently depending on past trajectory of the animal (Wood et al., Neuron 2000 27(3):623-33 or Pastalkova et al., Science, 321(5894):1322-7, 2008). This is highly relevant to the manuscript because sequence execution requires maintained history (which arm the mouse visited for far).
These studies do not exclude the possibility that IKD based mechanism is involved. However, place cell firing is well described in radial mazes, and they are thought to be implicated in the formation of sequence (e.g. through “phase precession” and “theta sequence”). If it is not possible to change the behavior study to better align with in vitro part, the authors could at least add or modify the simulation part to better integrate the 5-arm maze behavioral result by incorporating well described place cell and potentially time-cell activity in the model together with IKD-dependent dynamics.
Other concerns that I addressed were adequately addressed.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors addressed all my prior concerns

Reviewer #3

(Remarks to the Author)
Dear authors,
My concerns were all addressed by this round of revision. I hope the authors advance this field by conducting in vivo recordings in behaving animals in the future.
Best regards,

Jan. 15, 2026

Dear Editor,

We greatly appreciate invaluable and constructive comments from Editors and Reviewers. We also thank for their time and patience. We are re-submitting our manuscript which was substantially revised according to Reviewers' comments. The major improvements are as follows:

1. We confirmed that the aversive conditioning (single electrical shock) does not alter the CA3 ensemble size, the excitability of CA3 pyramidal cells, and inducibility of LTP of intrinsic excitability (**Figure S1C-D**) (our answer to 'Conceptual issues' #1 of Referee #1).

2. From analyses of the freezing levels and the freezing time courses in context B (context used for the second visit), we presented evidence supporting that animals are engaged in both acquisition of contextual information and retrieval of fear memory (**Figure S2**) (our answer to '*Figure-by-figure evaluation*' #3 of Referee #1).

3. To validate the simulation results, we theoretically deduced the conditions for hetero-association of CA3 neuronal ensembles and pattern completion-based recall of whole sequence of memories under the CA3 network of a rat scale (see **Supplementary Discussion**) (our answer to 'Conceptual issues' #3 of Referee #1).

4. Complying with the suggestion of Referee #3, we tested trace fear conditioning, a different temporal associative learning task, for Kcna2+/- mice. We presented results for this experiment (our answer to 'Major issue' #1 of Referee #3).

5. We presented the theoretical basis why all the CA3 pyramidal cells receive inputs from ensemble cells in the entorhinal cortex through perforant pathway (see **Supplementary Discussion**) (our answer to Major issue #4 of Referee #3)**.**

Below we made point-by-point responses to the referees' comments in blue fonts.

Sincerely yours,

Suk-Ho Lee, M.D., Ph.D.
Professor
Seoul National University College of Medicine
103 Daehak-ro, Jongno-gu, Seoul,
Seoul 03080, South Korea

leesukho@snu.ac.kr.

Dear Professor Lee,

Your manuscript entitled "The hippocampal CA3 area implements sequence learning of discontinuous episodes" has now been seen by 3 referees, whose comments are appended below. You will see from their comments copied below that while they find your work of potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be interested in considering a revised version that addresses these concerns.

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. In general, we would ask you to address all the referees' comments. In particular, please consider performing the additional behavioral and electrophysiological analyses and experiments mentioned by reviewers #1 and #3.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if you wish to discuss the revision or if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If you decide to submit a revised version, we ask that your manuscript complies with our editorial policies. Specifically:

For all graphs depicting a single point value (e.g., mean) with error bars, you must add individual data points or convert the graph to a boxplot or dot-plot to show data distribution.

It's mandatory to provide access to the numerical source data for graphs and charts either through a repository or by providing the data in a Supplementary Data file (in excel format).

All blots/gels must be accompanied by size markers in every figure panel. Uncropped and unedited blot/gel images must be included as Supplementary Figure(s) in the Supplementary Information pdf.

Please ensure that you have complied with the data deposition policies at the Nature Portfolio, please see here.

Please ensure that you have complied with our policies on research involving animals and humans, see here

Please follow the ARRIVE guidelines for reporting animal experiments. Please fully complete an ARRIVE checklist including both the essential and recommended set of items (adding information to the manuscript where needed) and upload this with your revised manuscript.

Please also see our revision checklist for guidance on formatting the manuscript and complying with our policies. A comprehensive guide to our formatting requirements for final submissions is also available for your reference here.

Please use the following link to submit your revised manuscript, point-by-point response to the referees' comments (which should be in a separate document to any cover letter) and any completed checklist:

https://mts-commsbio.nature.com/cgi-bin/main.plex?el=A6Cx2OyU4A1CZtE7I5A9ftdu5h2YjQhDOg7z26EVOr3mAZ

This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage first

We hope to receive your revised manuscript within 3 months, but appreciate that every situation is unique. Please take as long as necessary to address these concerns in full, including performing any additional experimental work required. We look forward to receiving your revised manuscript when it is ready and will not enforce any specific deadline. However, please bear in mind that if the revision process takes significantly longer than six months, we may take into account whether anything similar has been accepted for publication at Communications Biology or published elsewhere in the meantime.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Benjamin Bessieres, PhD

Senior Editor

Communications Biology

orcid.org/0000-0003-4660-1170

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Dear Authors,

I have carefully reviewed the manuscript and commend the authors for tackling a challenging and important problem: how CA3 intrinsic excitability plasticity may support the linking of experiences into sequences. This is a creative and ambitious study that integrates slice physiology, computational modeling, and behavior. The work is intriguing, but in its current form the manuscript requires major revisions before it can be considered for publication. Below I provide a structured assessment.

Summary of the manuscript

The authors examine bi-directional regulation of intrinsic excitability (IE) in CA3 pyramidal neurons. Using c-fos–based viral tagging, they show that ensemble neurons activated in a contextual fear conditioning (CFC) paradigm display reduced input conductance and higher excitability. With repeated exposure, subgroups emerge: some maintain high excitability, others revert to baseline, interpreted as PP-driven reset. They link these effects to Kv1.2/D-type K^+ current reduction.

A computational CA3 model incorporates these rules to demonstrate ensemble linking without saturation. Finally, in a five-arm radial maze task, CA3-Kcna2+/– mice perform normally on simple cue-guided conditions but are impaired under high probe-load conditions (C1P3), which the authors interpret as a sequence learning deficit.

The manuscript aims to connect cellular ion channel mechanisms to ensemble linking and sequence behavior.

Conceptual issues and concerns about interpretation

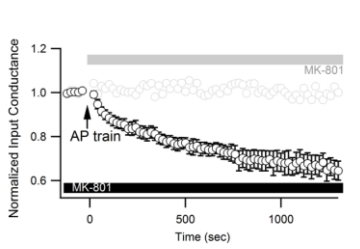
1. Aversive conditioning confound: All physiology derives from CFC with foot shock, yet the Discussion treats exposures as neutral. Stress strongly affects CA3 excitability, and freezing behavior is not reported.

> Thank you for this critical comment. The behavioral procedure for exposure of animals to a novel context (context A) adopted in Figures 1 to 5 was different from the standard contextual fear conditioning (CFC) in that we omitted the step for the animal to be habituated to the context A one day before conditioning. A single electrical shock was given in order to ensure that the animals give an attention to the novel context. To test if a single shock in context A has any effects on the CA3 ensemble size and the CA3 pyramidal cell excitability, we repeated the same novel context exposure experiments without a shock as shown in **Fig. S1C-D**. The animals which experienced a single electrical shock in context A were not different from non-shocked animals for the CA3 ensemble size (**Fig. S1-C**), the baseline and post-conditioning input conductance G_{in} (**Fig. S1-D**), implying that the presentation of a shock has little effect on the CA3 ensemble dynamics regulated by LTP-IE. Please see Fig. S1C-D, and line 156.

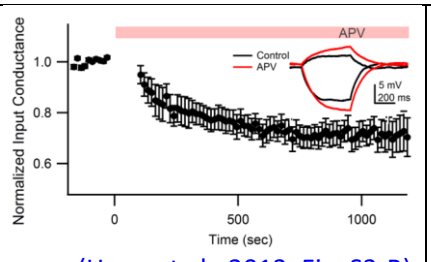
In addition, we analyzed the freezing behavior in context B during the second visit, which is shown as **Fig. S2**. Although the freezing in context B was higher compared to baseline, it was lower and delayed than that observed in animals subject to a standard contextual fear conditioning (line 183).

2. Synaptic vs intrinsic ambiguity: EPSP/EPSC changes could reflect synaptic AMPAR plasticity. AMPA/NMDA ratios were not measured, leaving open a purely synaptic explanation.

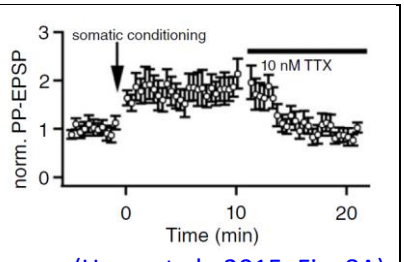
> Thank you for raising this thoughtful question. Previously we reported that LTP-IE is readily induced by somatic action potential (AP) trains in the presence of MK-801, a NMDA receptor (NMDAr) blocker (Fig. S2-A of Hyun et al., 2013)[1]. In the same study, we also tried but failed to test the effects of APV, another NMDAr blocker, on LTP-IE (Fig. S2-C of Hyun et al., 2013), because APV itself reduced the baseline input conductance (G_{in}) of CA3 pyramidal cells (CA3-PCs) from unknown reason (Fig. S2-B of Hyun et al, 2013) [1]. For your convenience, we replicated these results as the first and second figures shown below. In addition, MF-induced increase in PP-EPSP amplitudes was abolished in the presence of low concentration TTX (10 nM) that blocks dendritic Na^+ spikes (Fig. 8A of Hyun et al., 2015; replicated as the third figure below) [2], indicating that LTP-IE is mediated by lowering the threshold for dendritic Na^+ spikes rather than potentiation of NMDAr-mediated current.



(Hyun et al. 2013, Fig. S2-A)



(Hyun et al., 2013, Fig. S2-B)



(Hyun et al., 2015, Fig. 8A)

More critically, we have also tested the effects of NMDAr blockers (CPP, MK801 and APV) on MF conditioning-induced potentiation of PP-EPSPs in Fig. 4D of Hyun et al., 2015 (replicated as the figure shown below). In this figure, MF conditioning selectively potentiated PP-EPSP with little effects on PP-EPSC (panel *Ab* of Figure shown below). Among three different kinds of NMDAr blockers (CPP, MK801 and APV) we tested, MF-induced potentiation of PP-EPSP was abolished by APV, but not affected by CPP and MK-

801. The APV effect could be understood in light of our study in Hyun et al., 2013, in which we showed that APV reduced the baseline G_{in} from unknown reason.

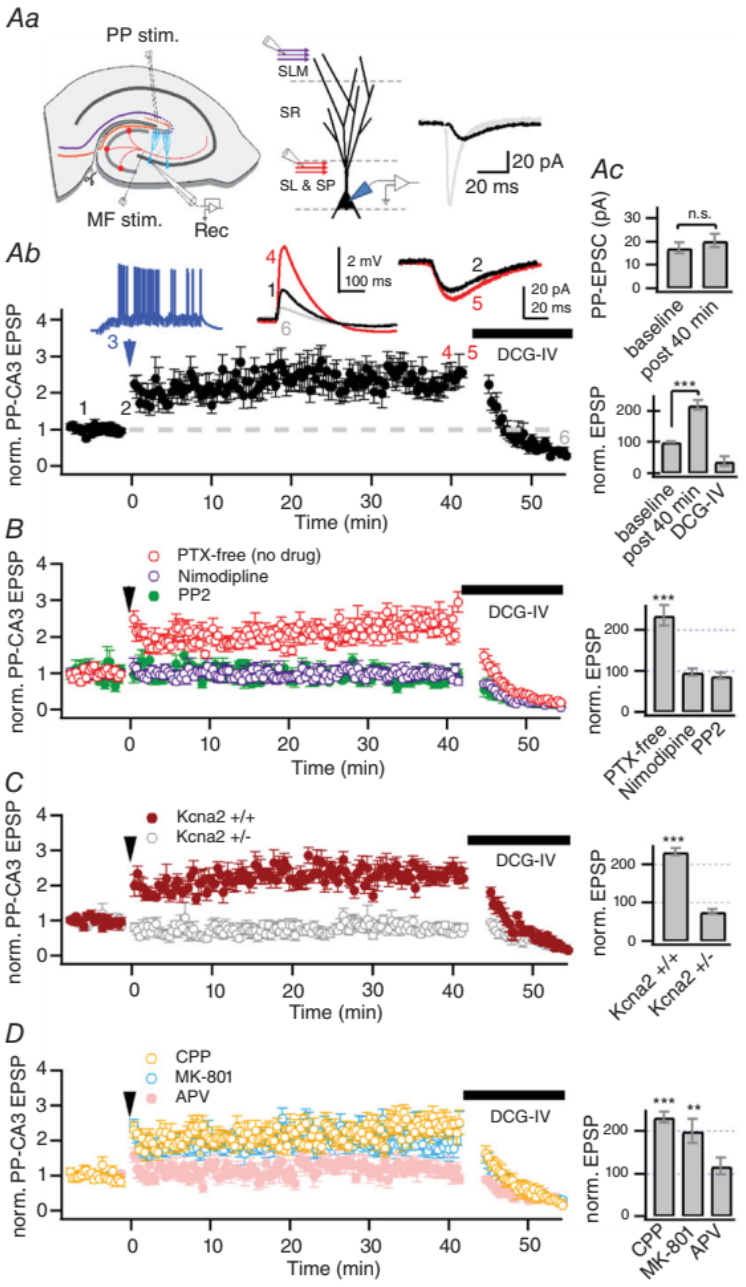


Figure 4. Downregulation of Kv1.2 mediates MF input-induced non-Hebbian heterosynaptic LTP of PP–CA3 EPSPs
Aa, left, experimental set-up. The incision was made along the hippocampal sulcus of the slice to prevent possible contamination of indirect cortical input. Stimulating electrodes were placed at the stratum lucidum and in close proximity to the hippocampal sulcus to activate mossy fibres (MF stim, red) and perforant path (PP stim, purple) inputs, respectively. Right, schematic diagram of synaptic inputs to apical dendrites of a CA3-PC. Inset: representative somatic recordings of EPSC evoked by stimulation of MF (grey) or PP (black trace) in the same CA3-PC. *b*, burst stimulation of MF induced LTP of PP–CA3 EPSPs. Stimulation of afferent MFs at 20 Hz for 2 s at $t = 0$ (blue arrow; MF conditioning) resulted in postsynaptic AP firing (blue trace in inset, denoted as '3'). Upper right two insets show representative PP-EPSP and PP-EPSC traces recorded at the time points indicated by the numbers (black, control; red, after MF conditioning; grey, DCG-IV). PP-EPSPs were attenuated by bath-applied DCG-IV to *ca* 30% of the baseline. *Ac*, summary of mean amplitudes of PP-EPSCs and PP-EPSPs before and 40 min after MF conditioning. *B*, MF conditioning-induced potentiation of PP-EPSPs occurs in PTX-free solution too, and was abolished by nimodipine (purple circles, 10 μM in the bath) and PP2 (green circles, 10 μM in the bath). *C*, the MF conditioning induced LTP of PP-EPSPs in CA3-PCs of WT mice, but not in those of *Kcna2* HT mice. *D*, the MF conditioning potentiated PP-EPSPs in the presence of CPP (yellow circles, 20 μM in the bath) or MK-801 (light blue circles, 40 μM in the bath), but not in the presence of APV (light red circles, 40 μM in the bath). Bar graphs in *B*–*D* summarize the effects of the drugs or mouse genotypes on MF conditioning-induced relative changes in PP-EPSP amplitudes at 40 min.

Therefore, it is unlikely that the increase in EPSP/EPSC ratio is mediated by an increase in NMDAr current. Nevertheless, complying with Reviewer's comment, we tried to measure PP-induced AMPA/NMDA current ratio before and after somatic conditioning. It was not trivial, however, to measure PP-induced NMDAr-mediated ESPCs (NMDA-EPSCs) because of a spatial voltage clamp issue. As we have shown in our previous study (Hyun et al., 2013), back-propagating APs are critical to induce LTP-IE, and thus we had to use K^+-based internal pipette solution. Under this internal K^+ conditions, it was not possible to holding the

membrane potential at positive potential to induce NMDA-EPSCs. Therefore, although we measured EPSCs at -20 mV of somatic potential in the presence of picrotoxin (GABAa blocker) and CNQX (AMPA/Kainate-R blocker), we cannot guarantee that our measurement was done at -20 mV at distal apical dendrites, in which PP synaptic sites are located. Moreover, because the effect of CNQX was not completely washed out, we inevitably measured AMPA- and NMDA-EPSC in different cells.

Therefore, you should assess our results shown below (**Fig. R1**), having these caveats in mind. We measured AMPA-EPSCs at holding potential of -70 mV in the presence of picrotoxin, and NMDA-EPSCs were measured at -20 mV in the presence of picrotoxin and CNQX (20 µM) using K-gluconate pipette solution. The induction of LTP-IE increased neither AMPA-EPSCs nor NMDA-EPSCs (5 neurons / 5 slices / 3 animals, For AMPA-EPSCs: 22.34 ± 5.55 pA vs. 22.46 ± 5.31 pA; For NMDA-EPSCs: 8.84 ± 2.01 pA vs. 8.72 ± 2.03 pA). Naturally AMPA/NMDA ratio was not altered by the LTP-IE induction (0.38 ± 0.03 vs. 0.39 ± 0.01; Mann-Whittney U = 6.00, p = 0.175; **Fig. R1A-B**). Although we found that there is no evidence for a change in AMPA/NMDA ratio after somatic conditioning, we did not add these results to the text of the present study because of afore-mentioned caveats.

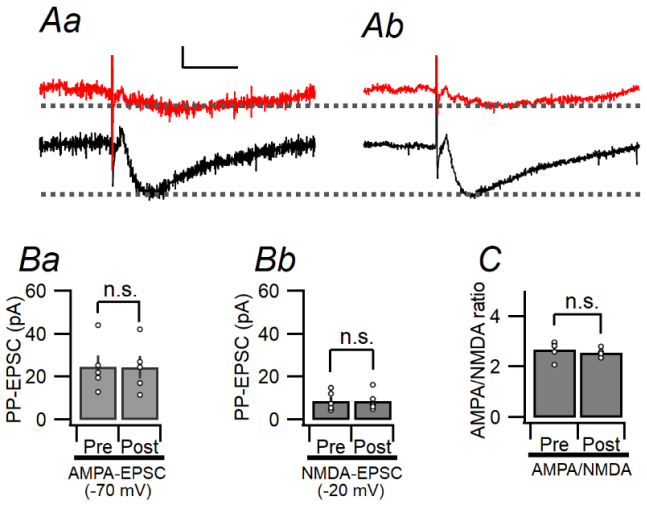


Figure R1. AMPA/NMDA ratio of PP-CA3 synapses before and after the somatic conditioning. **Aa-Ab.** Traces of NMDA-EPSC (red, measured at -20 mV) and AMPA-EPSC (black, measured at -70 mV) measured before (*Aa*) and after the somatic conditioning (*Ab*). **Ba-Bb.** Summary for peak AMPA-EPSC (Ba) and peak NMDA-EPSC (Bb) amplitudes before (pre) and after (post) somatic conditioning. **C.** AMPA/NMDA ratio of PP-CA3 synapses before (pre) and after (post) somatic conditioning.

3. Model limitations: The network model uses 10% PC–PC connectivity (vs <2% in vivo) and artificially accelerated IE kinetics. It illustrates feasibility but does not provide predictive validation.

> Thank you for pointing out this issue for validation of the CA3 model in our study. Below in **Table R1**, we compared the our simulation parameters with those of a real CA3 network of a rat [3]. The CA3 ensemble size (equivalent to an $\mathbf{M}_i$ subset in the simulation) was set to 5% of total number of CA3-PCs (N_{pc}) in the real CA3 network (according to Fig. S1-Cc). The number of CA3-PCs (N_{PC}) and the proportion of INs (N_{IN}) were set to the values that are generally accepted [3]. Under these conditions, the number of active presynaptic cells in a CA3 ensemble is estimated as 200 cells when the connectivity (p) is 2% (M = N_{pc} x 5% = 10^4, and M x p = 200)[4]. On the other hand, it was 20 cells (M p = 200 x 0.1) under our simulation conditions because M = 200 and connectivity (p) = 10%. Given that the mean strength of recurrent synaptic inputs is largely proportional to the number of active presynaptic cells, our simulation conditions for

pattern completion-based recall are fairly parsimonious compared to the real CA3 network of a rat scale.

Hetero-association of orthogonal ensembles can be tested by testing whether activation of the first memory ensemble, M_1, can trigger a recall of the entire sequence of memories, as shown in Fig. 7E. To evaluate the plausibility for this in the CA3 network of a rat scale, we roughly estimated the minimal proportion of primed CA3-PCs in an ensemble required to pattern completion-based recall of the next ensemble. Assuming that a CA3 ensemble is comprised of 5% of total number of CA3 principal cells (10^4 cells out of 2 x 10^5), and that recurrent synaptic conductance within an ensemble is two-times stronger than the baseline value (0.5 x 2 = 1 nS) [4], our calculation indicates that the proportion of primed PCs in an ensemble (that is M_i^*/M_i) required for activation of the next ensemble (M_{i+1}) is 6.4% of the given ensemble cells (M_i) (see ***Supplementary Discussion*** for details). In summary, if about 10% of PCs in each ensemble underwent LTP-IE, which is recruited to an M* subset, during encoding phase, hetero-association of two different ensembles is enabled. Then, during a recall phase, activation of entire sequence of memory ensembles can be triggered by PP or MF inputs that activate the first ensemble in a sequence.

For the accelerated IE kinetics, please see our answer to your comment on Figure 6 in 'Figure-by-figure evaluation'.

Table R1, Comparison of simulation parameters with those of the CA3 network of a rat scale.

	A rat CA3 network	**Simulation**
N_{PC}	200,000 [3]	1000
N_{IN} / N_{PC}	10-20%	13%
No. of CA3-PCs in an M subset (M/N_{PC})	10,000 (5%) [5]	200 (20%)
G_{MF}/G_{PP}	5 - 10 [6, 7]	10
Baseline / Potentiated G_{AC}	0.5 nS [4] / unknown	0.5 / 1 nS
A/C connectivity (p)	2% [4]	10%
No. of presynaptic PCs in an M subset (= M p)	200	20
τ_{STDP} at A/C synapse	40 ms [8]	40 ms
τ_{STDP} at PP synapse (LTD/LTP)	unknown	30 / 15 ms

N_{PC}, total number of pyramidal cells; N_{IN}, number of inter-neurons; G_{MF} /G_{PP}, conductance ratio of MF to PP synapses; G_{AC}, conductance of recurrent synapses; τ_{STDP}, time constant of STDP plot.

4. Behavioral interpretation: Only C1P3 genuinely requires sequence recall. C3P1 can be solved without true sequential memory. Analyses use only success/failure rates; no latency, trajectory, or trial-level mixed-

effects analyses are provided. Motivation and stress controls are absent.

1) Latency analysis: Complying with your suggestion, we analyzed the change of cue-to-reward latency for successful probe trials across training days (**Figure R2**). The latency was measured from the time of starting sound cue to the time getting water reward [Day, $F_{(4,26.266)} = 1.605$, $p = 0.203$; Genotype, $F_{(1,56.437)} = 2.207$, $p = 0.143$; Day x Genotype: $F_{(4,26.066)} = 1.030$, $p = 0.411$; LME model and simple effect analysis]. Although both genotype mice displayed a trend for reduction of latency across training days, only wild-type mice displayed a significant reduction on last two days (D4 and D5) compared to the initial latency (D1) [For WT, $F_{(4,25.369)} = 1.978$, $p = 0.128$; D1 vs D2, $p = 0.412$; D1 vs D3, $p = 0.418$; D1 vs D4, $p = 0.039$, D1 vs D5, $p = 0.033$; For Kcna2+/-, $F_{(4,25.369)} = 0.740$, $p = 0.574$; D1 vs D2, $p = 0.121$; D1 vs D3, $p = 0.366$; D1 vs D4, $p = 0.427$; D1 vs D5, $p = 0.251$; LME model and simple effect analysis].

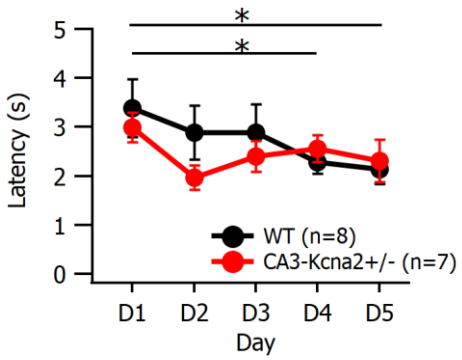


Figure R2, Change in cue-to-reward latency for successful probe trials across training days. Post-D1 (D2-D5) latencies were compared with the D1 latency for each genotype, and their p-values are indicated as an asterisk (*, $p < 0.05$).

2) Trajectory analyses: We compared the results for analysis of tower visits during probe trials of the C1P3 task on Day 5 in WT and CA3-Kcna2+/- mice in **Fig. 9Dc**. In this analysis, we compared the frequency of visits to the target tower in a given trial (T_1) with frequencies of visits to the target tower of previous and next trials (T_0 and T_2, respectively) or other towers (T_{others}). This analysis revealed that WT animals predominantly visited T1, whereas visits of Kcna2+/- mice to T1 were not significantly higher than those to any other towers (Fig. 9Dc). In addition, we added an exemplar movie for a mouse performing the spatial sequence task as **Supplementary Movie**.

3) Trial-level mixed effects analyses: We compared the mixed effects using LME model for the results of Fig. 9. Please see the legend of Figure 9.

4) We described, in Materials and Methods, how the motivation and stress of animals were controlled while they were performing sequence learning task. Line 819 (underlined).

5. Writing clarity: Critical details are sometimes obscured, for example using “CFC” in figure legends without spelling out “contextual fear conditioning.” This makes it too easy to miss that aversive shock was administered.

> Thank you for pointing out our carelessness. In the revised manuscript, we explicitly mentioned that a single electrical shock was routinely given in context A. Please see Line 118 and Line 168.

6. Reporting gaps: Viral titers are not provided, infection-level controls are missing, and most recordings were under PTX.

1) **Viral titer** was 4.8×10^{12} genome copies/ml. We added the titer in Line 691.

2) **Infection level control**: To measure the infection level in the rats infected with AAV-RAM-d2tTA-TRE-EGFP, which expresses EGFP under the control of modified cFos promoter (RAM), we induced a seizure by intraperitoneal injection of kainic acid (KA) under dox-free conditions two weeks after stereotaxic hippocampal injection of the same virus with the same titer as in Figure 1. One hour after a KA-induced seizure was induced, we sacrificed the animals, and counted EGFP(+) and EGFP(-) neurons in the same focal planes of hippocampal live slices under the epifluorescence imaging in combination with infrared-differential interference contrast (IR-DIC) imaging. The proportions of EGFP(+) cells in animals which visited a novel context and in animals experienced KA-induced seizure was 3% and 53%, respectively. These results suggest that the infection efficiency might be close to 50% assuming most CA3-PCs are activated under the KA-induced seizure. This notion is also supported by the expression of EGFP(+) cells induced by a novel context (3%) is about a half of the proportion of Arc(+) cells (5 to 7%; Fig. S1Cc and Eom et al. 2022). Please see Line 142, **Fig. S1B** and its legend.

Overall, while the dataset is rich, the claims currently overstep what the evidence directly supports.

Figure-by-figure evaluation

Figure 1 — Ensemble LTP-IE

Strength: clear demonstration of IE changes.

Concern: foot shock confound; recordings under PTX.

Needed: non-aversive controls, freezing data, intact inhibition recordings.

> We compared the size of CA3 ensemble in animal novel context with/without an electrical shock. As aforementioned, the size of CA3 ensemble activated by visiting a novel context was not different whether the novel context was aversive or not (**Fig. S1-C**). Moreover, we compared the baseline input conductance (G_{in}) and the inducibility of LTP-IE. The baseline and post-conditioning G_{in} values were not different between animals which experienced a single shock in the novel context or not (**Fig. S1-D**).

Figure 2 — GFP intensity

Strength: Alexa-594 normalization.

Concern: viral titer absent; GFP intensity confounded by infection load.

Needed: titer reporting, infection spread, and a constitutive GFP expression control to confirm GFP itself does not alter excitability.

> Please note that we did not use virus for the experiments in Figures 2 to 5. Instead, we used transgenic mice that express short half-life EGFP (shEGFP) under the control of c-fos promoter (denoted as *cfos-shEGFP mice*).

Figure 3 — Double-activated subgroups

Strength: identification of heterogeneous IE responses.

Concern: clustering choices not justified. Importantly, exposure to context B (highly similar to A) could represent a recall session, not a new encoding event, complicating interpretation.

Needed: validation of clustering, explicit treatment of context B as recall.

> We validated the k-means clustering by showing that the highest average silhouette width (ASW) was found at k = 2 (**Fig. S3**; Line 226). In addition, we thank for pointing out the critical issue that retrieval of fear memory may be involved in the double activation of ensemble cells in Figure 3.

It was inevitable to use the second context (context B) similar to the first context (context A) to increase the number of CA3 cells that are activated in both contexts (c.a. 50% of ensemble cells; Fig. 5 in Eom et al., 2022). We have tried to do the same experiments as those in Figure 3 using a completely distinct context (context C) instead of a similar context (context B). While doubly-activated cells [ctxAC(++) cells] were observed, their low density within a slice preparation precluded obtaining a sufficient number of successful patch-clamp recordings necessary for drawing statistically meaningful conclusions. This challenge was compounded by the inherently low yield recordings, typical of CA3 recording in acute brain slices. Therefore, to achieve a number of double-activated cells sufficient for patch recording experiments, we inevitably used a similar context (context B) instead of a distinct context (context C), compromising the assessment of cells doubly activated in two distinct contexts.

Nevertheless, we think that mice are engaged in both acquisition and retrieval of contextual information in context B, judging from the analysis of freezing behaviors of the animals in context B (**Fig. S2**). Please see line 181 (text for Fig. 2 in Results) and line 568 (Discussion subtitled "*Limitation and Weakness of the present study*").

Figure 4 — PP EPSP/EPSC

Strength: correlation with G_in.

Concern: lack of AMPA/NMDA ratios, PTX conditions.

Needed: AMPA/NMDA recordings, intact inhibition replications.

> As aforementioned, we have previous confirmed that CPP and MK801, NMDAr blockers, do not affect LTP-IE (Hyun et al., 2015). In addition, we performed additional experiments as shown in **Figure R1**.

Figure 5 — D-type K^+ current

Strength: plausible link to IE.

Concern: 4-AP not Kv1.2-specific; no dendritic evidence.

Needed: α-DTX, normalization, kinetics, dendritic validation.

> Please note that we used low concentration 4-aminopyridine (30 μM) for specific block of D-type K current. It is widely accepted that low 4-AP is specific blocker for D-type K current (Storm, 1988)[9]. In addition, we have previously shown that the activity-induced reduction of input resistance (G_{in}) is blocked by dendrodotoxin-I (DTX-I) but not by DTX-K, which are blocker of Kv1.1/2/6 and Kv1.1, respectively (Figure 4 in Hyun et al., 2013).[1] Moreover, Hyun et al (2103 and 2015) have shown that LTP-IE requires the dendritic Ca^{2+} signaling, dendritic Na^+ spikes, and intact presence of *Kcna2* gene.[1, 2]. We would appreciate your understanding that we tried to show reduction of D-type K current in CA3 ensemble cells rather than specifying Kv1.2 in Fig.5 of the present study.

Figure 6 — Model encoding

Strength: conceptual linkage.

Concern: unrealistic connectivity and kinetics; no data validation.

Needed: sensitivity analyses, slower IE implementation.

> As we mentioned above (our answer to 'conceptual issues' #3), we simulated the sequence encoding and recall under very parsimonious conditions compared to the real CA3 network in that the number of active presynaptic cells was only 10% of the real CA3 network (M x p = 20 in our simulation vs. 200 in the real CA3 network).

As you pointed out, both inter-event interval and the kinetics of intrinsic excitability (IE) changes were set to be fast in our simulation in order to reduce the computation time *in silico*.

We would like to remind you, however, of the principle for the induction of LTP-IE and its depotentiation as described in Results (line 356, underlined): "*Coincident increases in cytosolic [Ca^{2+}] and [Zn^{2+}] induce LTP-IE, while a [Ca^{2+}] increase alone restores intrinsic excitability to baseline*". In other words, restoration of the high excitability state of a CA3-PC which underwent LTP-IE in the previous event depend not on spontaneous decay of IE (at least until 90 min as shown in Figs. 1 and 3) but on the arrival of PP inputs in the next event, which evoke a Ca^{2+} transient devoid of Zn^{2+} signalling. Because restoration of high

excitability state does not spontaneously decay but is enforced by PP synaptic inputs in the next event, it is clear that lengthening inter-event intervals does not affect the simulation results.

Figure 7 — Model recall

Strength: illustrates sequence recall.

Concern: simplifications limit realism.

Needed: simulations with realistic IE timescales, predictions testable in physiology.

> We estimated the number of active PCs required for an ensemble to activate the next ensemble in the subsection of **Supplementary Discussion,** entitled "*Conditions for hetero-association and trans-ensemble excitation under the CA3 network of a rat scale*".

Figure 8 — Task design

Strength: clear schematics.

Concern: C3P1 does not test sequence memory; probe failures rescued with cues; only males; no motivation/anxiety controls.

Needed: sequence randomization, removal of within-run rescue, trajectory/latency analyses.

> We tried a sequence randomization in the C1P3 scheme as you suggested, but mice were not able to learn this task at all. Indeed, as shown in Fig 9Da, it took five days for wild-type mice to reach a learning criterion even for a single sequence. Along this line, we had to rescue the probe failure with cues in order not to confuse the mice under training.

For maintaining motivation without affecting general performance, we were very careful for restriction of water to the mice while maintaining their body weight at 80% of normal value (Line 818).

We confirmed that the anxiety level of *Kcna2*+/- mice is normal using an elevated plus maze in our previous study (Figure 3e of Eom et al. 2022)[5].

Figure 9 — Behavior results

Strength: WT vs mutants diverge under C1P3.

Concern: C3P1 not diagnostic; small/uneven Ns; binary data not analyzed with mixed-effects models.

Needed: logistic mixed-effects analysis, effect sizes, behavioral controls, and acute CA3 manipulations or Kv1.2 rescue to establish necessity.

> As we discussed below in response to '*Additional recommendation*', we think that acute overexpression or knock-down experiments are not appropriate for testing the role of LTP-IE in the spatial sequence learning task.

In addition, we re-analyzed our results using a linear mixed effect (LME) model throughout our article (Figures 1 to 5 and Figure 9, and Supplementary Figures 1 to 3). We described statistic parameters such as effect sizes (generally, F-value) and p-value throughout our article.

Minor issues

1) Spell out "CFC" and provide freezing curves.

> CFC stands for contextual fear conditioning. For experiments of Figures 1 to 5, however, we have omitted the habituation step, which is the first step for the standard CFC protocol on Day 1. As mentioned above, in the present study, a single electrical shock was given to ensure that mice turn an attention to the context, not to train animals for CFC. Therefore, we did not use the term, CFC, any more in the revised Ms. Instead, we used exposure to a novel context with a single electrical shock instead of CFC.

2) Report viral titers and infection spread.

> We reported viral titer in line 690. Please note that viral infection was used only in Figure 1 for comparing excitability of ensemble and non-ensemble cells in rats. In other figures we used transgenic mice (cfos-shEGFP mice). In Figure 1, we always performed patch recordings within one or two slices from the focus of viral injection. Because infected cells do not constitutively express fluorescent proteins, we could not routinely check the infection spread. The range of infection spread would be critical for analyzing animal behaviors. But it was not our purpose of the experiments in Figure 1.

3) Clarify animal-level Ns and apply hierarchical statistics.

> We re-analyzed our data using the linear mixed effect (LME) model to count in the variance between experimental animals and slices. The main statistical parameters are presented in the attached Supplementary Data File in a Microsoft Excel format.

4) Replicate key findings under intact inhibition.

> Previously, we have confirmed that MF conditioning can induce potentiation of PP-EPSP in the absence of picrotoxin (Figure 4B of Hyun et al., 2015)[2]. This figure is replicated in our answer to 'Conceptual issues' #2.

5) State sex of animals consistently.

> Only male animals were used in the present study. We explicitly described it in Materials and Methods.

6) Model connectivity should reflect biological values or provide sensitivity testing.

> As aforementioned, our simulation was done fairly under parsimonious conditions compared to real CA3 network of a rat scale. Because it was not practical to simulate sequence learning on the CA3 network of a real rat scale, we estimated what proportion of CA3 ensemble cells is needed to undergo LTP-IE in order for the ensemble to be hetero-associated with the next ensemble in the CA3 network of a rat scale. We found that LTP-IE in about 10% of ensemble cells is sufficient for hetero-association of two CA3 neuronal ensembles representing temporally adjacent two events. Please see ***Supplementary Discussion***, entitled "*Conditions for hetero-association and trans-ensemble excitation under the CA3 network of a rat scale*".

Additional recommendations

1. The Discussion should include a Limitations and Weaknesses section, explicitly acknowledging the aversive conditioning context, possible synaptic contributions, illustrative modeling, and behavioral strategy alternatives.

> We added "*Limitations and weaknesses of the present study*" to Discussion. In addition, please see our replies to your comments in 'Limitation and Weakness' below.

Crucially, the study lacks causal manipulations:

A gain-of-function experiment (e.g., artificially boosting CA3 excitability plasticity and testing sequence behavior) would establish sufficiency.

A loss-of-function experiment (acute CA3 silencing or Kv1.2 rescue/knockdown during behavior) would test necessity. These are essential to establish a causal role for CA3 IE in sequence learning.

> The neuronal excitability is a key regulator for recruiting principal cells to a memory trace. For example, if the excitability is artificially increased in a subset of principal cells using over-expression of CREB or dominant negative KCNQ2 or hM3Dq DREADD, they were preferentially allocated to memory trace or to an engram encoding fear memory, while principal cells overexpressing Kir2.1 were preferentially excluded from memory engrams [10, 11]. Furthermore, the effects of CREB overexpression was abolished by co-overexpression of Kir2.1 [11]. Artificial manipulation of neuronal excitability using gain or loss of function mutation of ion channels in a subset of principal cells has been used for elucidating the key factors that determine which neurons will be recruited to an engram that encodes a simple association memory [12]. However, the same method cannot be applied to our behavioral paradigm in which multiple neuronal ensembles may have to be timely recruited to encode sequence of multiple memories, because

uncontrolled silencing or overexpression of *Kcna2* in a subset of principal cells using viral vectors will preferentially recruit or exclude infected cells to memory ensembles. Therefore we cannot test the essential role of LTP-IE in sequential learning using viral knockdown or overexpression of Kv1.2.

Different from viral manipulation of cell excitability, hetero-knock out (KO) of *Kcna2* causes little heterogeneity in the baseline excitability among principal cells and specifically abolishes LTP-IE in CA3 pyramidal cells. For example, *Kcna2* hetero-KO mice were normal in one-trial contextual fear conditioning and pre-exposure fear contextual fear conditioning, implying intact CA3 recurrent network supporting pattern completion-based recall [5]. These features of *Kcna2*+/- mice provide us a precious opportunity which allows us to investigate the specific behavioral consequences to the lack of LTP-IE with minimal effects on neuronal excitability and without causing population heterogeneity in the excitability, which may result from uncontrolled overexpression or knock-down of Kcna2. We therefore respectfully ask that the requirement to perform corresponding additional experiments be waived.

Limitation and Weakness

1) aversive conditioning context

> As aforementioned, we repeated the experiments of Fig. 1 without a shock in context A. We found that the CA3 ensemble size, the baseline excitability of CA3-PCs, and their LTP-IE inducibility are not different whether the animals experienced a shock or not in context A [**Fig. S1C-D** and associated text, Line 156].

2) possible synaptic contribution to LTP-IE

> We have previously ruled out the possible contribution of NMDAr to MF-induced potentiation of PP-EPSPs (Figure 4D of Hyun et al., 2015, replicated as the second figure in our reply to '*Conceptual issues*' #2).

3) illustrative modeling

> We added Figure 10 to the revised manuscript.

4) behavioral strategy alternatives

> As Review #3 suggested in his/her Major issue #1, we tested wild-type and Kcna2+/- mice for trace fear conditioning (tFC), another temporal associative learning task. Unfortunately, we failed to find any difference in the performance of tFC between these two genotypes. Please see our reply to the comment of Reviewer #3 for details.

Conclusion and recommendation

This is an innovative and ambitious manuscript, but several conceptual, methodological, and interpretational issues need to be addressed. The physiology is intriguing, the modeling creative, and the behavioral design promising, yet the manuscript currently over-claims and omits critical controls.

Recommendation: Major revision. Addressing the aversive conditioning confound, adding synaptic assays, improving behavioral analysis, clarifying reporting, and including causal gain- and loss-of-function experiments will be necessary to substantiate the central claims.

In closing

This manuscript reflects substantial effort and creativity. With additional rigor, transparency, and acknowledgment of limitations, it has the potential to become a field-shaping contribution. I look forward to a strengthened revision.

Thanks,

Reviewer #2 (Remarks to the Author):

The authors of this manuscript use electrophysiology, modeling and behavioral approaches with the goal to understand how intrinsic plasticity determined by changes in IKD in CA3 neurons might contribute to sequence learning. The results might be potentially interesting; however I find that the manuscript is very difficult to read, especially in its first part, because of the way the findings are currently presented.

Some of the issues are detailed below.

1)The numbers for all the experimental (sub)groups presented should be included to be able to better evaluate the validity of the findings in function of the sample size.

> We added the number of cells, slices, and animals for all experiments.

2) It would be useful to have a summary figure to explain the framework, including the synaptic inputs and the kind of plasticity they undergo as they pertain to the various experiments, especially for researchers who are not deeply familiar with the specific topic.

> We added a summary cartoon as Figure 10.

3) The authors should clearly state their definition of CA3 ensemble cells in the beginning, as the term is mentioned but not defined in the introduction (line 93). I assume that it means CA3 neurons that are activated in a specific context, but this in not clearly stated.

> We added following statement in Introduction: “A memory ensemble is a subset of principal cells that are co-activated while a memory is represented in a network.” (Line 94)

4) Some of the names of the experimental subgroups appear to be convoluted, making it difficult to follow the presentation of the findings (see for example ctxAB+, ctxAB++ as well ctxAB(++/1) and ctxAB(++/2) also mentioned below).

> We apologize for the confusion. In the revised Ms, we made efforts for clearer description for ctxAB(+) and ctxAB(++), which represents cell subgroups displaying low and high EGFP expression (Fig. 3Be). CtxAB(++) were further separated into two subgroups depending on their baseline input conductance (G_{in}) (Fig. 3Cc). Please see line 228 (underlined) and line 231 (underlined).

5) In Fig. 3, were ctxAB(++/1) and ctxAB(++/2) cells recorded from the same or different animals?

> Both category cells were found in the same animals.

Minor:

Abstract lines 41-42: something is missing in this sentence.

> We rephrased this sentence as “Here we show that CA3 ensemble cells activated by visiting a novel context display high excitability, while the excitability states of those activated by re-visiting a similar context diverge into high and normal excitability states.”. Please see Line 42.

Introduction, lines 59-63: reference should be added for these statements.

> We cited a following book chapter in the text: “Amaral D, Lavenex P. Hippocampal neuroanatomy. The hippocampus book. New York, NY, US: Oxford University Press; 2007. p. 37-114.” (Line 61, Reference #6)

Results: is there a reason why the experiments in Fig. 1 are performed in rats whereas the rest are in mice?

> Previously our *in vitro* slice electrophysiology experiments were mainly performed in rats, and thus we first confirmed LTP-IE *in vivo* in the rats. However, we need to repeat it in mice because CA3-Kcan2+/- rats, which lack LTP-IE, were not available for behavioral tests.

Results, lines 145-148: it is unclear how these sentences refer to Fig. 2, since only fluorescence data is presented in this Figure.

> We are sorry for this confusion. However, we think this introductory remark necessary for readers to understand why we need to do the experiment shown in Figure 2. To highlight our purpose for the experiments in Figure 2, we re-phrased the introductory statement (Line 175).

Results, line 134: is ctxAB(++) here a typo? Because the two context experiments are not introduced until later.

> Yes, it is. We apologize our mistake. We deleted it.

Results, Figure 3Be: the term shEGFP(++) is not introduced anywhere in the manuscript.

> We defined shEGFP(++) in Line 231 (underlined).

Results, lines 236-238: the sentence is unclear.

> We corrected this sentence as “*Because ctxAB(++/1) and ctxAB(++/2) cell groups exactly overlapped with*

the ctxAB(++) cell subsets displaying high and low EPSP/EPSC ratio, respectively, ctxAB(++) cells exhibiting high EPSP/EPSC ratio could be regarded as ctxAB(++/1) cell, and the other subset as ctxAB(++/2) cell (Fig. 4Ab).”. Please see line 280 (underlined)

Methods, line 538: the sentence about glutathione is unclear, since it is not mentioned elsewhere.

> We deleted the sentence because we have already mentioned the composition of internal solution in the same paragraph.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors tested their hypothesis that Kv1.2 channels in CA3 pyramidal cells contribute to sequence learning. The group focuses on the role of Kv1.2 channels in cellular excitability for years. In this manuscript they expand their previous in vitro work to test their hypothesis in behaving mice and a computational model. They first demonstrate that exposure of mice to a novel context increases cellular excitability in a Kv1.1 and Kv1.2 channel dependent manner. Second, authors propose a computational model where this mechanism can form a sequential synaptic link. Third, they show that mice with knockdown of these channels are impaired in a sequence memory task. The combination of these results is novel and interesting, addressing the behavioral relevance to the potassium channel dependent plasticity of intrinsic excitability. These studies that aim to understand the role of molecular level mechanism in cognition have potentials to develop future treatments for cognitive deficits. However, there are several major issues as I describe below.

Major:

1. The behavioral task that the authors used does not support strongly the mechanism the authors are interested in is involved. This is because there is no temporal discontinuity in this task. Firing of place cells in CA3 that fire at different locations on the maze are known to overlap in time as the animals run. They can therefore be linked through a simple STDP mechanism without involving IKD mechanism that the authors focus on this paper. Better tasks are, for example, trace conditioning or sequence of odor presentations separated by temporal gaps, which have been shown to depend on hippocampus.

> Thank you for these thoughtful suggestions. We tried inter-trial intervals longer than 5 s (10 or 15 s), but could not get a learning curve even in the WT mice. Lengthening the inter-trial interval disrupted the task performance, probably because mice failed to maintain their focus on the task goal during the long interval. We think that mice might not be sufficiently attentive to the spatial sequence learning task when longer intervals were used. We have also attempted to test the Eichenbaum-style odor sequence learning task in mice, but it was not successful even in the wild-type mice, probably from the same reason why mice failed to learn the spatial learning tasks with an inter-trial interval of 10 s or longer. From the same reason, most sequence learning tasks might have been tested in rats, not in mice [13] [14] [15]. Because *Kcna2* KO rats were not available, we had to test mice for the spatial sequence learning task.

Please note that inter-trial intervals were set to 5 s. During encoding phase of the training, mice should remain idle for 5 s until next target cue is presented. Given the time constant of spike-timing-dependent potentiation (STDP) is less than 100 ms, the 5 s interval is long sufficient for prevent synaptic LTP through NMDAr-dependent STDP. Although two adjacent memories can be recalled in a very short time once an animal has learnt a sequence, it is not the case while animals encode a novel sequence.

As Reviewer suggested, we have also tested the possibility for LTP-IE to contribute to trace fear conditioning (tFC) (**Fig. R3**). However, CA3-*Kcna2*+/- mice were normal in the tFC task, consistent with Suh

and Tonegawa (2011)[16]. It needs further study why CA3 does not contribute to tFC.

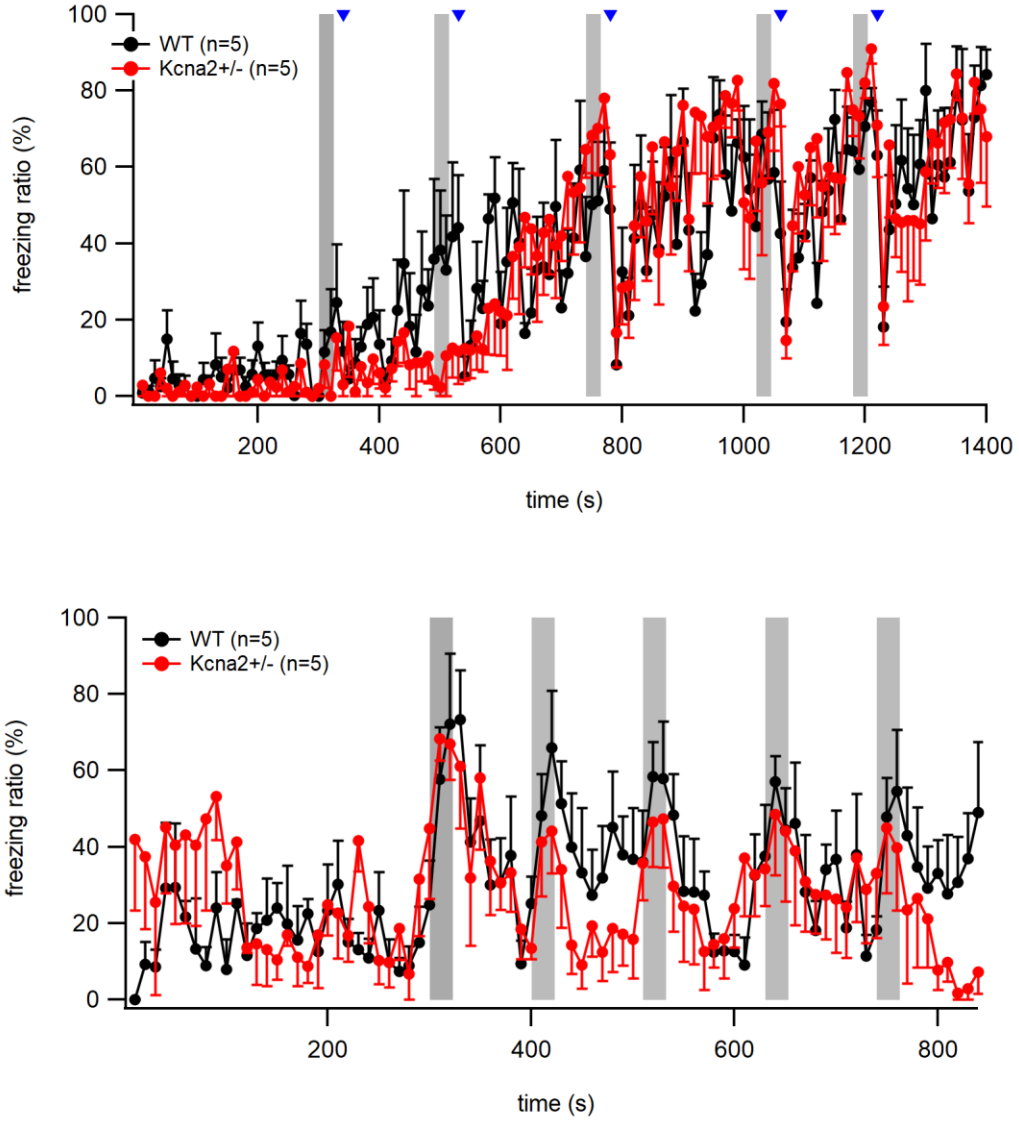


Figure R3, Time courses of freezing ratio during TFC (*upper*, Day1) and tone test (*lower*, Day2) on the next day in Kcna2+/- mice (red) and their wildtype littermates (black). Time periods for presentations of 20 s tone were indicated as shaded boxes. On day 1, 0.5 mA shock was given for 2 s after the end of each tone (*triangles*). On the next day, responses to the same tone of the mice were tested in a distinct context from that of day 1. Freezing ratio was measured every 10 s as a proportion of motionless duration. The methods employed for trace fear conditioning is described in detail in Lee et al. (2004)[17].

Methods for Trace Fear conditioning: The fear conditioning was performed in a context A, which is a mice conditioning cage (18 cm wide × 18 cm long × 28 cm high; H10-11M-TC, Coulbourn Instruments, USA) located inside a sound-attenuating box (SCITECH, Korea) with an LED lamp. An auditory cue was presented via a speaker module (H12-01R, Coulbourn Instruments, USA) located on one side of the

conditioning chamber. The floor of the conditioning cage consisted of stainless-steel bars to deliver a footshock through an electrical stimulator (H13-15-220, Coulbourn Instruments). Context B is rectangular (16 cm wide × 16 cm long × 20 cm high) and consists of a white acrylic floor, and a white acrylic wall with a black check pattern. The experimental protocol was performed by Ethovision XT 13 software. To quantify freezing behaviors, a python-based open source code ezTrack (Pennington, 2019) was used. The parameters we used for this analysis were a motion cutoff of 20, a freezing threshold of 30, and a minimum freeze duration of 30 samples. The behavior was recorded with a CCD camera.
CA3-Kcna2 +/- mice and wild-type mice were used for trace fear conditioning experiments. In trace fear conditioning (day 1), the mice received five CS-US pairings which were delivered at 300, 490, 740, 1020, 1180 sec. The CS-US pairing consisted of tones (2.5kHz, 20 s, 75 dB) and a footshock (0.5 mA, 2 s). CS and US are separated by a 20 s trace interval. The intertrial intervals were 220 ± 60 s. After each test, the context A was cleaned with 70% alcohol. The next day, in the tone test (day 2), the mice received five presentations of tones (20 s, 2.5 kHz, 75 dB) which were delivered at 300, 400, 510, 630, 740 sec in context B. The intertrial intervals of tone presentations were 110 ± 10 s. After each test, the chamber was cleaned with 1% ammonia.

2. Grouping of cells and statistical analysis in ex vivo experiments are not scientifically appropriate in Fig1-4.

2-1. In Fig1, to address the effect of cell activation, which is marked by the EGFP expression, Gin of all EGFP- and EGFP+ has to be compared. It is not scientifically correct to separate observed results into groups based on observed measurements (analysis is circular). The interpretation that “EGFP(-/low) cells may represent memory ensemble cells that are not infected by AAV” might be true. But it is not scientific to separate observed data points into groups based on such speculation. Authors need evidence.

> We appreciate the reviewer's concern that separating EGFP(-) cells into high and low G_{in} groups based on observed measurements may constitute circular reasoning. To resolve this, we have employed silhouette analysis[18], a gold-standard unsupervised clustering validation method, to objectively determine whether the EGFP(-) population contains distinct subgroups. Based on this concept, we re-wrote the text describing Figure 1B. Please see line 125-135.

2-2. Similarly, in Fig 3, the interpretation “Because cells with distinctly high G/R ratio were observed only in the mice which visited both context A and B, the ctxAB(++) cells may be activated in both contexts, while cells in the other group were activated once.” is a speculation. It is not correct to separate the results into two groups based on the value you observed from ctxAB group based on such speculation. It is obvious if one separates observed group into high and low data points that you will get significant difference between those groups (which is shown in Fig3 Be ctxAB+ vs ctxAB++ comparison with *** $p<0.0001$). Similarly, if you take the high value group of ctxAB group and compared to the ctxA+ group, finding out that ctxAB++ group is higher (which is shown in Fig3Be as well) is nothing to do with the fact that the animal went through ctx A and B, it could just be because authors took high values from ctxAB group.

2-3. In Fig3 C to E, it is necessary to have the **ctxAB group** which include all ctxAB+, ctxAB++1 and ctxAB++2 groups together. If further sub-categorization is needed, authors can add extra analysis where the authors focus on sub grouping of ctxAB group with careful justification.

> For comments #2-2 and #2-3, we acknowledge the reviewer's concern that categorizing G/R ratios of ctxAB cells could appear as speculation. Following the same rigorous validation of clustering as in Figure 1, we applied silhouette analysis for *k*-means clustering to objectively determine the optimal clustering of ctxAB cells. Please see line 226-229 and Fig. S3.

3. In Fig2, how do we know that this difference of fluorescence intensity distribution is not simply due to the time from the last exposure to a context? To address this, context B only exposure followed by ex vivo experiment 60 min later is needed.

> We have already shown that fluorescence intensities of EGFP(+) cells are not different at 60 and 90 min after single exposure to a novel context (context A) in Figure 1B (red and blue circles) and in Figure 3Be [ctxA60(+) vs. ctxA90(+)]. Consistent with this result, Figure 3 in Chowdhury & Caroni (2018; www.doi.org/10.1038/s41467-018-06516-3) have shown that the fraction of cFos(+) cells remains unchanged until 180 min in the hippocampal CA3 area [19]. Therefore, it is unlikely that the time from the last exposure to a context caused the difference of fluorescence intensity distribution between ctxA and ctxAB mice.

4. In the modeling study, authors used PP input to all groups (M1-M4). This is misleading considering the fact that the authors stress "orthogonal" memory representation. Since the model heavily rely on this, it should be justified based on empirical data.

> We added theoretical plausibility that all the CA3 principal cells may receive synaptic inputs from ensemble cells in the entorhinal cortex (EC) via perforant pathway (PP) in **Supplementary Discussion** (subtitled "*Plausibility for all the CA3 principal cells to receive ensemble inputs from EC via PP*"). Assuming random connections between EC and CA3 principal cells, this calculation predicts that most CA3-PCs receive synaptic inputs from more than 300 ensemble cells of the EC. Therefore, all CA3-PCs have a potential to be re-activated by PP during memory retrieval as long as the PP synaptic weight is sufficiently high. For a CA3-PC to have high PP synaptic weights, a help of concomitant MF inputs is essential because MF inputs provide depolarization required for inducing PP-LTP (homosynaptic LTP at PP synapses)[7]. Moreover, the present and our previous studies showed that, if the MF inputs are sufficiently strong, the postsynaptic CA3-PC undergo LTP-IE, and can be also recruited for encoding of the next event at PP synapses. Therefore, the recruitment of a CA3-PC to a PP synaptic ensemble is dictated by sparse MF inputs. Along this line, MF inputs during an encoding phase is essential for a CA3 network to perform pattern separation. Once the synaptic weights at PP and A/C synapses are modified with a help of MF input, however, memory ensembles encoding similar memories can be distinctly retrieved by PP inputs alone, as predicted by Treves and Rolls (1994)[20] and evidenced by Lee and Kesner (2004)[21].

4. Different mechanism that are proposed to support temporally separated events should be mentioned in the introduction and discussion (e.g. persistent activity, replay, short-term synaptic plasticity). In addition, other mechanisms involved in activity induced change in excitability should also be mentioned (e.g. H-current).

> Thank you for this suggestion. We added two paragraphs to Discussion section entitled "*Activity-dependent regulation of ion channels in the CA3 area*" and "*Other mechanisms for hetero-association of temporally separated events*". Please see Line 553 and 598.

Minor:

It should be written in the abstract and introduction that labeling of active cells in context exposure was done in this study.

In the abstract, the statement that "Here we show that the high excitability of CA3 ensemble cells in the animals experienced an event can be normalized by a subsequent event." is not supported. This, as the authors discuss in the Discussion, is an interpretation.

> We rephrased the statement as "Here we show that CA3 ensemble cells activated by visiting a novel context display high excitability, while the excitability states of those activated by re-visiting a similar context diverge into high and normal excitability states."

L 52: English needs to be checked by a native speaker. For example, "capture hypothesis may link two neuronal ensembles" is probably wrong because "hypothesis" does not "link" memories.

> We apologize for the confusion. We meant "*synapse tagging and capture hypothesis"* that was first proposed by Frey and Morris (1997)[22] and has been supported by subsequent papers [reviewed in Rogerson et al (2014)] [23]. To avoid confusion, we italicized the phrase "*synapse tagging and capture hypothesis"* in the text.

L 118: "Whole-cell patch" -> "Whole-cell patch recording"?

> We corrected it to "Formation of the whole-cell patch configuration" (Line 123).

L 134: ctxAB(++) appears first time here and it is not clear what it means and how this is relevant.

> We apologize our mistake. The "in ctxAB(++) cells" was erroneously inserted, and thus we deleted it.

Fig1.B: It should be indicated from which group each EGFP- cells came from. Did the EGFP(-/low) group came from specific group (60, 90 or 180 min)?

> We explicitly described that all GFP(-) cells were obtained from animals of 60 min. Please see the legend of Fig. 1B.

Fig1.Cc: Is it a mistake that EGFP(-/high) shows n.s.?

> The horizontal range bar was erroneously drawn. Thank you for pointing this out. We corrected it in Fig. 1Cc.

Fig1.B: If the authors' main claim is that inclusion of cells into assembly causes the reduction of Gin, then they should show statistical comparison of the 60 and 90 min group compared to control and EGFP- groups (without separating them into low and high).

> Complying with your suggestion, we added a statement for comparison of the 60 and 90 min group with the control and EGFP(-) groups (Line 125). As you expected, we found no difference when EGFP(-) cells were not separated. Nevertheless, given that the infection rate is about 50% (Fig. S1B) and that there was no EGFP(-) displaying low G_{in} in the c*fos*-shEGFP-transgenic mice (Fig. 3Cf), it is likely that the EGFP(-/low) cells may represent memory ensemble cells that are not infected by AAV (Line 142).

L 216: underwent -> undergone

> Thank you. We corrected it as you suggested.

L 261: It helps if there is a summary statement of this section at the end.

> As you suggested, we added a summary statement (Line 308).

Fig. 6 and 7: The text goes back and forth between Fig6 and 7. Modify the figures or the text to avoid back and forth.

> Figure 6 and associated text were corrected as you suggested.

L 344: The explanation of the task is hard to understand. Especially, it is not clear enough for the first time reader how the sequence is implemented. Instead of "A 'run' was completed, when a mouse visited four towers in a sequence (Fig. 8A).", it would be better to write something like: "A sequence animal needed to learn was defined by a 'run' which consisted of four trials (i.e. visiting of four towers) ".

> We appreciate your kind suggestion. We corrected the statement as you suggested (Line 410).

L 348: "guided run" should be defined.

> Guided run is defined as a run in which all trials are guided by light cue. We defined in the text (Line 405).

L 390: This possibility that some ctxAB cells that went back to normal excitability level is a natural flow of

the paper which is expected to be mentioned in the results section than the discussion section.

> Thank you for your suggestion. Complying with it, we added a summary paragraph (Line 308).

L417: “are lacking”

> We corrected it as you suggested.

L423: “little has been understood”

> We corrected it as you suggested.

L425: Logic around here is difficult to follow. Especially, it is often unclear if the word “overlap” is used for temporal overlap or spatial (neuronal representation) overlap . This is the case also in Line 36 “non-overlapping representation of memories”.

> We meant an overlap between ensembles representing different memories. In Abstract, we used more formal word, ‘orthogonal’, instead of ‘non-overlapping’. We explicitly defined ‘ensemble overlap’ in the context of the paragraph as “ensembles representing temporally separated episodes to share a number of principal cells (called ensemble overlap)” (Line 473).

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Dear Editor

We greatly appreciate constructive comments from Reviewers as well as their time and patience. We also thank to Editor for giving us an opportunity to revise our manuscript. We are sending our manuscript revised according to Reviewers' comments. The major improvements are as follows:

1. Complying with Reviewer #2, we thoroughly revised the summary cartoon in Figure 10.

2. Complying with Reviewer #3, we newly added a simulation, in which activation of place cells during inter-event intervals was considered (Figure S4). We also discussed the possibility for the role of sequential firings of place or time cells in encoding of sequential episodes.

We believe that the present study may present a possible cellular and synaptic mechanism underlying hippocampus-dependent replaying of event sequence, which was recently discovered in rodents and humans.[1, 2]

Sincerely yours,

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Reviewer #1 (Remarks to the Author):

Dear authors,

The revised version of the manuscript has answered my concerns to a large extent. I have no further

comments. Congratulations on your interesting study.

Best Regards,

Reviewer #2 (Remarks to the Author):

The authors addressed most of the issues I raised in my prior review; however, there are still a couple of issues.

MAJOR

Figure 10 does not clarify and, if possible, makes the summary more confusing.

What I was asking was to identify which inputs (MF, vs PP vs A/C) are causing what type of plasticity and in which order. This is missing in the diagram. In addition, only event 1 and 2 of the four events in the diagram are described in the figure legend. Finally, at line 1039, does non-MF inputs refer to A/C input, PP inputs or both?

> We appreciate this constructive comment. Complying with your suggestion, we thoroughly revised the Fig. 10 to append external synaptic inputs to the model network and consequent modification of recurrent synapses. In addition, we re-wrote the figure legend to cover all events (Event 1~4) and thus to explain whole processes of sequential memory formation in the CA3 network.

> The non-MF inputs refer to PP inputs in the line 1309 (line 1039 may be a typo of line 1309). We replaced “non-MF inputs” with “PP inputs” in the revised Ms.

MINOR

Fig. 2 does not report the number of samples in this data set. In all other data sets, the number of samples is reported in the figure legends, except for Fig. 5 where they are reported in the text. This seems an odd choice; for consistency, it may be better to report them all in the figure legends.

> The sample size of the dataset in Fig. 2 was added to the figure legend as (number of cells/number of slices/number of animals) in the same way as in other figures.

> The number of datasets for Fig. 5 has been deleted from the text, and moved to the figure legend.

Reviewer #3 (Remarks to the Author):

The first major concern I raised in the initial review remains: there is still a substantial gap between the behaviour task and the remaining. Increased excitability in the in vitro study was examined in relation to a novel context and aversive stimulation, neither of which is relevant in the 5-arm maze task. While the 5-arm maze task involves a sequence, the in vitro work used a novel context as the induction for IKD dependent mechanisms; therefore, it does not provide evidence that increased excitability occurs when the mice visited each arm or when the light cue was turned on. My previous mention of trace fear conditioning was to potentially address this exact point. The authors have, however, indicated that trace fear conditioning was tested but was unaffected.

> We agree with the Reviewer's concern that there is a large gap between our *ex vivo* and behavioral studies. However, it was not feasible to test the excitability changes of CA3-PCs during the five-arm maze. The exposure to a novel context with a foot-shock was employed in the present study, because this was a simple and practical way to test excitability changes of CA3-PCs *ex vivo*. Although we did not directly test excitability changes during the five-arm maze task, salient events in our five-arm maze task, such as reward delivery and the light cue, may engage hippocampal plasticity-related processes in light of a previous study in the CA1[3]. We agree, however, that this remains indirect, and we have revised the text to clarify this limitation.

In the revision, the authors pointed out that the change in increased excitability was not mainly due to the footshock, but rather the new context. I find it difficult to reconcile the precisely timed input in the model (which induced increased excitability) with context exposure. As far as we currently know, context exposure lacks such precisely timed, distinct inputs. Such time-constrained stimuli are more consistent with a footshock. This, unfortunately, represents a new mismatch that I noticed in this revision.

> We imagine that not only temporally discrete stimuli but also contextual inputs may induce an the excitability change in CA3-PCs as long as they induce MF-induced burst firing in CA3-PCs in light of our

previous *in vitro* studies. It remains to be tested, however, whether visiting a reward zone (a target tower) in the five-arm maze can induce LTP-IE in CA3-PCs as we assumed in the simulation. To confirm this view, we may need *in vivo* patch-clamp study on CA3-PCs in freely moving animals. Because it is not trivial, this question should be addressed as a further study in the future.

The authors claimed that the 5-second temporal gap between trials provides a discontinuity that requires mechanisms longer-lasting than NMDA-dependent synaptic plasticity. However, studies indicate that place cells remain active during immobility (Hok et al, Journal of Neuroscience, 27 (3) 472-482, 2007). It has also been shown that "time cells" can bridge such temporal gaps of up to 5–10 seconds (Pastalkova et al., Science, 321(5894):1322-7, 2008). These indicates that hippocampal activity could bridge the 5-second gap. Therefore, the situation is unlike the simulation, where there is no input or firing in the hippocampus for 5 seconds. If this activity maintains the information about the current arm, the association between the current arm and the next light cue might be done with NMDA-dependent time scale. In fact, it has been reported that place cells and time cells can fire differently depending on past trajectory of the animal (Wood et al., Neuron 2000 27(3):623-33 or Pastalkova et al., Science, 321(5894):1322-7, 2008). This is highly relevant to the manuscript because sequence execution requires maintained history (which arm the mouse visited for far).

These studies do not exclude the possibility that IKD based mechanism is involved. However, place cell firing is well described in radial mazes, and they are thought to be implicated in the formation of sequence (e.g. through "phase precession" and "theta sequence"). If it is not possible to change the behavior study to better align with in vitro part, the authors could at least add or modify the simulation part to better integrate the 5-arm maze behavioral result by incorporating well described place cell and potentially time-cell activity in the model together with IKD-dependent dynamics.

> We are grateful for this insightful comment. As Reviewer suggested, we examined how place cell firings during inter-event intervals influence on encoding of sequence events in our network model. To simulate encoding of a sequential events in the presence of place cells firing, each inter-event interval was intervened by eight place fields, and each place field was represented by firing of 40 CA3-PCs, which is one-fifth of an M subset in size. The simulation results described in the text (line 438) associated with Figure S4, and discussed the possibility for the role of time and place cells in hetero-association of episodic memories (line 573). As newly described in Discussion, recent studies revealed that animals and humans

replay a sequence of multiple episodes during or after non-spatial tasks requiring memory of event sequence [1] [2]. It remains to test whether our synaptic mechanism may underlie such replay of non-spatial tasks.

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In accordance with the guidelines of the Communications Biology editorial department, our point-by-point responses to all issues raised by the Reviewers are summarized below. Please note that all Reviewers' comments are included here.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Dear Authors,

I have carefully reviewed the manuscript and commend the authors for tackling a challenging and important problem: how CA3 intrinsic excitability plasticity may support the linking of experiences into sequences. This is a creative and ambitious study that integrates slice physiology, computational modeling, and behavior. The work is intriguing, but in its current form the manuscript requires major revisions before it can be considered for publication. Below I provide a structured assessment.

Summary of the manuscript

The authors examine bi-directional regulation of intrinsic excitability (IE) in CA3 pyramidal neurons. Using c-fos–based viral tagging, they show that ensemble neurons activated in a contextual fear conditioning (CFC) paradigm display reduced input conductance and higher excitability. With repeated exposure, subgroups emerge: some maintain high excitability, others revert to baseline, interpreted as PP-driven reset. They link these effects to Kv1.2/D-type K^+ current reduction.

A computational CA3 model incorporates these rules to demonstrate ensemble linking without saturation. Finally, in a five-arm radial maze task, CA3-Kcna2+/– mice perform normally on simple cue-guided conditions but are impaired under high probe-load conditions (C1P3), which the authors interpret as a sequence learning deficit.

The manuscript aims to connect cellular ion channel mechanisms to ensemble linking and sequence behavior.

Conceptual issues and concerns about interpretation

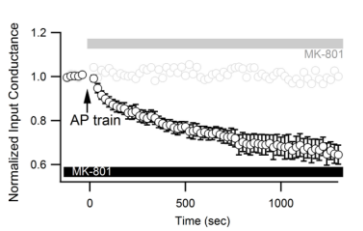
1. Aversive conditioning confound: All physiology derives from CFC with foot shock, yet the Discussion treats exposures as neutral. Stress strongly affects CA3 excitability, and freezing behavior is not reported.

> Thank you for this critical comment. The behavioral procedure for exposure of animals to a novel context (context A) adopted in Figures 1 to 5 was different from the standard contextual fear conditioning (CFC) in that we omitted the step for the animal to be habituated to the context A one day before conditioning. A single electrical shock was given in order to ensure that the animals give an attention to the novel context. To test if a single shock in context A has any effects on the CA3 ensemble size and the CA3 pyramidal cell excitability, we repeated the same novel context exposure experiments without a shock as shown in **Fig. S1C-D**. The animals which experienced a single electrical shock in context A were not different from non-shocked animals for the CA3 ensemble size (**Fig. S1-C**), the baseline and post-conditioning input conductance G_{in} (**Fig. S1-D**), implying that the presentation of a shock has little effect on the CA3 ensemble dynamics regulated by LTP-IE. Please see Fig. S1C-D, and line 156.

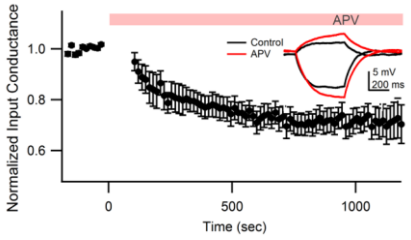
In addition, we analyzed the freezing behavior in context B during the second visit, which is shown as **Fig. S2**. Although the freezing in context B was higher compared to baseline, it was lower and delayed than that observed in animals subject to a standard contextual fear conditioning (line 183).

2. Synaptic vs intrinsic ambiguity: EPSP/EPSC changes could reflect synaptic AMPAR plasticity. AMPA/NMDA ratios were not measured, leaving open a purely synaptic explanation.

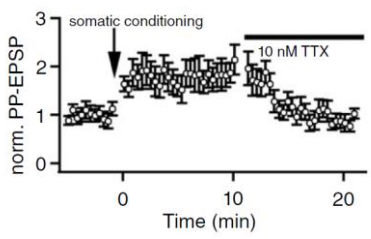
> Thank you for raising this thoughtful question. Previously we reported that LTP-IE is readily induced by somatic action potential (AP) trains in the presence of MK-801, a NMDA receptor (NMDAr) blocker (Fig. S2-A of Hyun et al., 2013)[1]. In the same study, we also tried but failed to test the effects of APV, another NMDAr blocker, on LTP-IE (Fig. S2-C of Hyun et al., 2013), because APV itself reduced the baseline input conductance (G_{in}) of CA3 pyramidal cells (CA3-PCs) from unknown reason (Fig. S2-B of Hyun et al, 2013) [1]. For your convenience, we replicated these results as the first and second figures shown below. In addition, MF-induced increase in PP-EPSP amplitudes was abolished in the presence of low concentration TTX (10 nM) that blocks dendritic Na^+ spikes (Fig. 8A of Hyun et al., 2015; replicated as the third figure below) [2], indicating that LTP-IE is mediated by lowering the threshold for dendritic Na^+ spikes rather than potentiation of NMDAr-mediated current.



(Hyun et al. 2013, Fig. S2-A)



(Hyun et al., 2013, Fig. S2-B)



(Hyun et al., 2015, Fig. 8A)

More critically, we have also tested the effects of NMDAr blockers (CPP, MK801 and APV) on MF conditioning-induced potentiation of PP-EPSPs in Fig. 4D of Hyun et al., 2015 (replicated as the figure shown below). In this figure, MF conditioning selectively potentiated PP-EPSP with little effects on PP-EPSC (panel *Ab* of Figure shown below). Among three different kinds of NMDAr blockers (CPP, MK801 and APV) we tested, MF-induced potentiation of PP-EPSP was abolished by APV, but not affected by CPP and MK-801. The APV effect could be understood in light of our study in Hyun et al., 2013, in which we showed that APV reduced the baseline G_{in} from unknown reason.

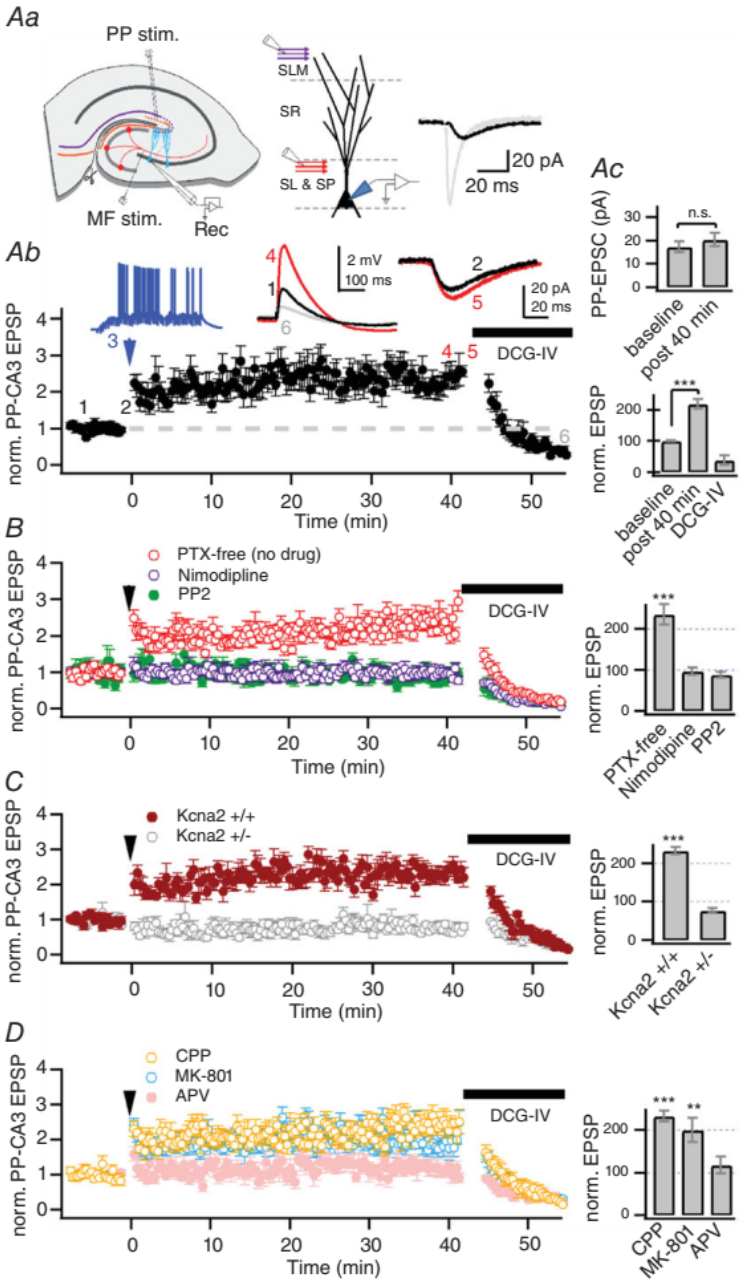


Figure 4. Downregulation of Kv1.2 mediates MF input-induced non-Hebbian heterosynaptic LTP of PP–CA3 EPSPs
Aa, left, experimental set-up. The incision was made along the hippocampal sulcus of the slice to prevent possible contamination of indirect cortical input. Stimulating electrodes were placed at the stratum lucidum and in close proximity to the hippocampal sulcus to activate mossy fibres (MF stim, red) and perforant path (PP stim, purple) inputs, respectively. Right, schematic diagram of synaptic inputs to apical dendrites of a CA3-PC. Inset: representative somatic recordings of EPSC evoked by stimulation of MF (grey) or PP (black trace) in the same CA3-PC. *b*, burst stimulation of MF induced LTP of PP–CA3 EPSPs. Stimulation of afferent MFs at 20 Hz for 2 s at $t = 0$ (blue arrow; MF conditioning) resulted in postsynaptic AP firing (blue trace in inset, denoted as '3'). Upper right two insets show representative PP-EPSP and PP-EPSC traces recorded at the time points indicated by the numbers (black, control; red, after MF conditioning; grey, DCG-IV). PP-EPSPs were attenuated by bath-applied DCG-IV to *ca* 30% of the baseline. *Ac*, summary of mean amplitudes of PP-EPSCs and PP-EPSPs before and 40 min after MF conditioning. *B*, MF conditioning-induced potentiation of PP-EPSPs occurs in PTX-free solution too, and was abolished by nimodipine (purple circles, 10 μM in the bath) and PP2 (green circles, 10 μM in the bath). *C*, the MF conditioning induced LTP of PP-EPSPs in CA3-PCs of WT mice, but not in those of *Kcna2* HT mice. *D*, the MF conditioning potentiated PP-EPSPs in the presence of CPP (yellow circles, 20 μM in the bath) or MK-801 (light blue circles, 40 μM in the bath), but not in the presence of APV (light red circles, 40 μM in the bath). Bar graphs in *B*–*D* summarize the effects of the drugs or mouse genotypes on MF conditioning-induced relative changes in PP-EPSP amplitudes at 40 min.

Therefore, it is unlikely that the increase in EPSP/EPSC ratio is mediated by an increase in NMDAr current.

Nevertheless, complying with Reviewer's comment, we tried to measure PP-induced AMPA/NMDA current ratio before and after somatic conditioning. It was not trivial, however, to measure PP-induced NMDAr-mediated ESPCs (NMDA-EPSCs) because of a spatial voltage clamp issue. As we have shown in our previous study (Hyun et al., 2013), back-propagating APs are critical to induce LTP-IE, and thus we had to use K^+-based internal pipette solution. Under this internal K^+ conditions, it was not possible to holding the membrane potential at positive potential to induce NMDA-EPSCs. Therefore, although we measured EPSCs at -20 mV of somatic potential in the presence of picrotoxin (GABAa blocker) and CNQX (AMPA/Kainate-R blocker), we cannot guarantee that our measurement was done at -20 mV at distal apical dendrites, in which PP synaptic sites are located. Moreover, because the effect of CNQX was not completely washed out, we inevitably measured AMPA- and NMDA-EPSC in different cells.

Therefore, you should assess our results shown below (**Fig. R1**), having these caveats in mind. We measured AMPA-EPSCs at holding potential of -70 mV in the presence of picrotoxin, and NMDA-EPSCs were measured at -20 mV in the presence of picrotoxin and CNQX (20 μM) using K-gluconate pipette solution. The induction of LTP-IE increased neither AMPA-EPSCs nor NMDA-EPSCs (5 neurons / 5 slices / 3 animals, For AMPA-EPSCs: 22.34 ± 5.55 pA vs. 22.46 ± 5.31 pA; For NMDA-EPSCs: 8.84 ± 2.01 pA vs. 8.72 ± 2.03 pA). Naturally AMPA/NMDA ratio was not altered by the LTP-IE induction (0.38 ± 0.03 vs. 0.39 ± 0.01; Mann-Whittney U = 6.00, p = 0.175; **Fig. R1A-B**). Although we found that there is no evidence for a change in AMPA/NMDA ratio after somatic conditioning, we did not add these results to the text of the present study because of afore-mentioned caveats.

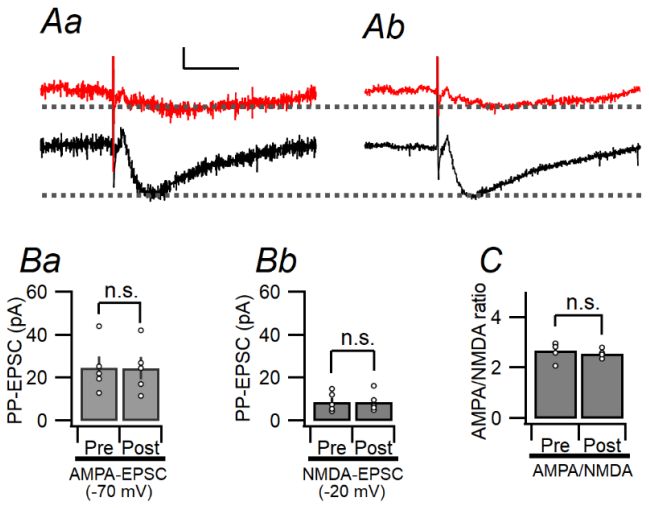


Figure R1. AMPA/NMDA ratio of PP-CA3 synapses before and after the somatic conditioning. **Aa-Ab.** Traces of NMDA-EPSC (red, measured at -20 mV) and AMPA-EPSC (black, measured at -70 mV) measured before (*Aa*) and after the somatic conditioning (*Ab*). **Ba-Bb.** Summary for peak AMPA-EPSC (Ba) and peak NMDA-EPSC (Bb) amplitudes before (pre) and after (post) somatic conditioning. **C.** AMPA/NMDA ratio of PP-CA3 synapses before (pre) and after (post) somatic conditioning.

3. Model limitations: The network model uses 10% PC–PC connectivity (vs <2% in vivo) and artificially accelerated IE kinetics. It illustrates feasibility but does not provide predictive validation.

> Thank you for pointing out this issue for validation of the CA3 model in our study. Below in **Table R1**, we compared the our simulation parameters with those of a real CA3 network of a rat [3]. The CA3 ensemble size (equivalent to an $\mathbf{M_i}$ subset in the simulation) was set to 5% of total number of CA3-PCs (N_{pc}) in the real CA3 network (according to Fig. S1-Cc). The number of CA3-PCs (N_{PC}) and the proportion of INs (N_{IN})

were set to the values that are generally accepted [3]. Under these conditions, the number of active presynaptic cells in a CA3 ensemble is estimated as 200 cells when the connectivity (p) is 2% ($M = N_{pc} \times 5\% = 10^4$, and $M \times p = 200$)[4]. On the other hand, it was 20 cells ($M\ p = 200 \times 0.1$) under our simulation conditions because M = 200 and connectivity (p) = 10%. Given that the mean strength of recurrent synaptic inputs is largely proportional to the number of active presynaptic cells, our simulation conditions for pattern completion-based recall are fairly parsimonious compared to the real CA3 network of a rat scale.

Hetero-association of orthogonal ensembles can be tested by testing whether activation of the first memory ensemble, M_1, can trigger a recall of the entire sequence of memories, as shown in Fig. 7E. To evaluate the plausibility for this in the CA3 network of a rat scale, we roughly estimated the minimal proportion of primed CA3-PCs in an ensemble required to pattern completion-based recall of the next ensemble. Assuming that a CA3 ensemble is comprised of 5% of total number of CA3 principal cells (10^4 cells out of 2×10^5), and that recurrent synaptic conductance within an ensemble is two-times stronger than the baseline value ($0.5 \times 2 = 1$ nS) [4], our calculation indicates that the proportion of primed PCs in an ensemble (that is M_i^*/M_i) required for activation of the next ensemble (M_{i+1}) is 6.4% of the given ensemble cells (M_i) (see ***Supplementary Discussion*** for details). In summary, if about 10% of PCs in each ensemble underwent LTP-IE, which is recruited to an M* subset, during encoding phase, hetero-association of two different ensembles is enabled. Then, during a recall phase, activation of entire sequence of memory ensembles can be triggered by PP or MF inputs that activate the first ensemble in a sequence.

For the accelerated IE kinetics, please see our answer to your comment on Figure 6 in 'Figure-by-figure evaluation'.

Table R1, Comparison of simulation parameters with those of the CA3 network of a rat scale.

	A rat CA3 network	**Simulation**
N_{PC}	200,000 [3]	1000
N_{IN} / N_{PC}	10-20%	13%
No. of CA3-PCs in an M subset (M/N_{PC})	10,000 (5%) [5]	200 (20%)
G_{MF}/G_{PP}	5 - 10 [6, 7]	10
Baseline / Potentiated G_{AC}	0.5 nS [4] / unknown	0.5 / 1 nS
A/C connectivity (p)	2% [4]	10%
No. of presynaptic PCs in an M subset (= M p)	200	20
τ_{STDP} at A/C synapse	40 ms [8]	40 ms
τ_{STDP} at PP synapse (LTD/LTP)	unknown	30 / 15 ms

N_{PC}, total number of pyramidal cells; N_{IN}, number of inter-neurons; G_{MF}/G_{PP}, conductance ratio of MF to PP synapses; G_{AC}, conductance of recurrent synapses; τ_{STDP}, time constant of STDP plot.

4. Behavioral interpretation: Only C1P3 genuinely requires sequence recall. C3P1 can be solved without true sequential memory. Analyses use only success/failure rates; no latency, trajectory, or trial-level mixed-effects analyses are provided. Motivation and stress controls are absent.

1) Latency analysis: Complying with your suggestion, we analyzed the change of cue-to-reward latency for successful probe trials across training days (**Figure R2**). The latency was measured from the time of starting sound cue to the time getting water reward [Day, $F_{(4,26.266)} = 1.605$, $p = 0.203$; Genotype, $F_{(1,56.437)} = 2.207$, $p = 0.143$; Day x Genotype: $F_{(4,26.066)} = 1.030$, $p = 0.411$; LME model and simple effect analysis]. Although both genotype mice displayed a trend for reduction of latency across training days, only wild-type mice displayed a significant reduction on last two days (D4 and D5) compared to the initial latency (D1) [For WT, $F_{(4,25.369)} = 1.978$, $p = 0.128$; D1 vs D2, $p = 0.412$; D1 vs D3, $p = 0.418$; D1 vs D4, $p = 0.039$, D1 vs D5, $p = 0.033$; For Kcna2+/-, $F_{(4,25.369)} = 0.740$, $p = 0.574$; D1 vs D2, $p = 0.121$; D1 vs D3, $p = 0.366$; D1 vs D4, $p = 0.427$; D1 vs D5, $p = 0.251$; LME model and simple effect analysis].

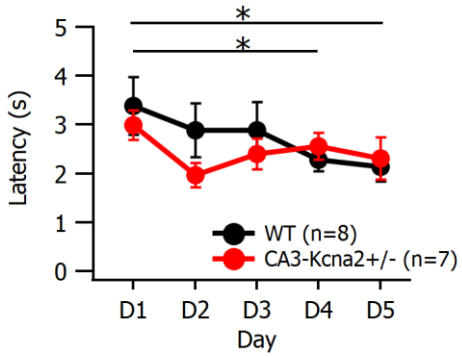


Figure R2, Change in cue-to-reward latency for successful probe trials across training days. Post-D1 (D2-D5) latencies were compared with the D1 latency for each genotype, and their p-values are indicated as an asterisk (*, $p < 0.05$).

2) Trajectory analyses: We compared the results for analysis of tower visits during probe trials of the C1P3 task on Day 5 in WT and CA3-Kcna2+/- mice in **Fig. 9Dc**. In this analysis, we compared the frequency of visits to the target tower in a given trial (T_1) with frequencies of visits to the target tower of previous and next trials (T_0 and T_2, respectively) or other towers (T_{others}). This analysis revealed that WT animals predominantly visited T1, whereas visits of Kcna2+/- mice to T1 were not significantly higher than those to any other towers (Fig. 9Dc). In addition, we added an exemplar movie for a mouse performing the spatial sequence task as **Supplementary Movie**.

3) Trial-level mixed effects analyses: We compared the mixed effects using LME model for the results of Fig. 9. Please see the legend of Figure 9.

4) We described, in Materials and Methods, how the motivation and stress of animals were controlled while they were performing sequence learning task. Line 819 (underlined).

5. Writing clarity: Critical details are sometimes obscured, for example using “CFC” in figure legends without spelling out “contextual fear conditioning.” This makes it too easy to miss that aversive shock was administered.

> Thank you for pointing out our carelessness. In the revised manuscript, we explicitly mentioned that a single electrical shock was routinely given in context A. Please see Line 118 and Line 168.

6. Reporting gaps: Viral titers are not provided, infection-level controls are missing, and most recordings were under PTX.

1) **Viral titer** was 4.8×10^{12} genome copies/ml. We added the titer in Line 691.

2) **Infection level control**: To measure the infection level in the rats infected with AAV-RAM-d2tTA-TRE-EGFP, which expresses EGFP under the control of modified cFos promoter (RAM), we induced a seizure by intraperitoneal injection of kainic acid (KA) under dox-free conditions two weeks after stereotaxic hippocampal injection of the same virus with the same titer as in Figure 1. One hour after a KA-induced seizure was induced, we sacrificed the animals, and counted EGFP(+) and EGFP(-) neurons in the same focal planes of hippocampal live slices under the epifluorescence imaging in combination with infrared-differential interference contrast (IR-DIC) imaging. The proportions of EGFP(+) cells in animals which visited a novel context and in animals experienced KA-induced seizure was 3% and 53%, respectively. These results suggest that the infection efficiency might be close to 50% assuming most CA3-PCs are activated under the KA-induced seizure. This notion is also supported by the expression of EGFP(+) cells induced by a novel context (3%) is about a half of the proportion of Arc(+) cells (5 to 7%; Fig. S1Cc and Eom et al. 2022). Please see Line 142, **Fig. S1B** and its legend.

Overall, while the dataset is rich, the claims currently overstep what the evidence directly supports.

Figure-by-figure evaluation

Figure 1 — Ensemble LTP-IE

Strength: clear demonstration of IE changes.

Concern: foot shock confound; recordings under PTX.

Needed: non-aversive controls, freezing data, intact inhibition recordings.

> We compared the size of CA3 ensemble in animal novel context with/without an electrical shock. As aforementioned, the size of CA3 ensemble activated by visiting a novel context was not different whether the novel context was aversive or not (**Fig. S1-C**). Moreover, we compared the baseline input conductance (G_{in}) and the inducibility of LTP-IE. The baseline and post-conditioning G_{in} values were not different between animals which experienced a single shock in the novel context or not (**Fig. S1-D**).

Figure 2 — GFP intensity

Strength: Alexa-594 normalization.

Concern: viral titer absent; GFP intensity confounded by infection load.

Needed: titer reporting, infection spread, and a constitutive GFP expression control to confirm GFP itself does not alter excitability.

> Please note that we did not use virus for the experiments in Figures 2 to 5. Instead, we used transgenic mice that express short half-life EGFP (shEGFP) under the control of c-fos promoter (denoted as *cfos-shEGFP mice*).

Figure 3 — Double-activated subgroups

Strength: identification of heterogeneous IE responses.

Concern: clustering choices not justified. Importantly, exposure to context B (highly similar to A) could represent a recall session, not a new encoding event, complicating interpretation.

Needed: validation of clustering, explicit treatment of context B as recall.

> We validated the k-means clustering by showing that the highest average silhouette width (ASW) was found at k = 2 (**Fig. S3**; Line 226). In addition, we thank for pointing out the critical issue that retrieval of fear memory may be involved in the double activation of ensemble cells in Figure 3.

It was inevitable to use the second context (context B) similar to the first context (context A) to increase the number of CA3 cells that are activated in both contexts (c.a. 50% of ensemble cells; Fig. 5 in Eom et al., 2022). We have tried to do the same experiments as those in Figure 3 using a completely distinct context (context C) instead of a similar context (context B). While doubly-activated cells [ctxAC(++) cells] were observed, their low density within a slice preparation precluded obtaining a sufficient number of successful patch-clamp recordings necessary for drawing statistically meaningful conclusions. This challenge was compounded by the inherently low yield recordings, typical of CA3 recording in acute brain slices. Therefore, to achieve a number of double-activated cells sufficient for patch recording experiments, we inevitably used a similar context (context B) instead of a distinct context (context C), compromising the assessment of cells doubly activated in two distinct contexts.

Nevertheless, we think that mice are engaged in both acquisition and retrieval of contextual information in context B, judging from the analysis of freezing behaviors of the animals in context B (**Fig. S2**). Please see line 181 (text for Fig. 2 in Results) and line 568 (Discussion subtitled "*Limitation and Weakness of the present study*").

Figure 4 — PP EPSP/EPSC

Strength: correlation with G_in.

Concern: lack of AMPA/NMDA ratios, PTX conditions.

Needed: AMPA/NMDA recordings, intact inhibition replications.

> As aforementioned, we have previous confirmed that CPP and MK801, NMDAr blockers, do not affect LTP-IE (Hyun et al., 2015). In addition, we performed additional experiments as shown in **Figure R1**.

Figure 5 — D-type K^+ current

Strength: plausible link to IE.

Concern: 4-AP not Kv1.2-specific; no dendritic evidence.

Needed: α-DTX, normalization, kinetics, dendritic validation.

> Please note that we used low concentration 4-aminopyridine (30 μM) for specific block of D-type K current. It is widely accepted that low 4-AP is specific blocker for D-type K current (Storm, 1988)[9]. In addition, we have previously shown that the activity-induced reduction of input resistance (G_{in}) is blocked by dendrodotoxin-I (DTX-I) but not by DTX-K, which are blocker of Kv1.1/2/6 and Kv1.1, respectively (Figure 4 in Hyun et al., 2013).[1] Moreover, Hyun et al (2103 and 2015) have shown that LTP-IE requires the dendritic Ca^{2+} signaling, dendritic Na^+ spikes, and intact presence of *Kcna2* gene.[1, 2]. We would appreciate your understanding that we tried to show reduction of D-type K current in CA3 ensemble cells rather than specifying Kv1.2 in Fig.5 of the present study.

Figure 6 — Model encoding

Strength: conceptual linkage.

Concern: unrealistic connectivity and kinetics; no data validation.

Needed: sensitivity analyses, slower IE implementation.

> As we mentioned above (our answer to 'conceptual issues' #3), we simulated the sequence encoding and recall under very parsimonious conditions compared to the real CA3 network in that the number of active presynaptic cells was only 10% of the real CA3 network (M x p = 20 in our simulation vs. 200 in the real CA3 network).

As you pointed out, both inter-event interval and the kinetics of intrinsic excitability (IE) changes were set to be fast in our simulation in order to reduce the computation time *in silico*.

We would like to remind you, however, of the principle for the induction of LTP-IE and its depotentiation as described in Results (line 356, underlined): "*Coincident increases in cytosolic [Ca^{2+}] and [Zn^{2+}] induce LTP-IE, while a [Ca^{2+}] increase alone restores intrinsic excitability to baseline*". In other words, restoration of the high excitability state of a CA3-PC which underwent LTP-IE in the previous event depend not on spontaneous decay of IE (at least until 90 min as shown in Figs. 1 and 3) but on the arrival of PP inputs in the next event, which evoke a Ca^{2+} transient devoid of Zn^{2+} signalling. Because restoration of high excitability state does not spontaneously decay but is enforced by PP synaptic inputs in the next event, it is clear that lengthening inter-event intervals does not affect the simulation results.

Figure 7 — Model recall

Strength: illustrates sequence recall.

Concern: simplifications limit realism.

Needed: simulations with realistic IE timescales, predictions testable in physiology.

> We estimated the number of active PCs required for an ensemble to activate the next ensemble in the subsection of **Supplementary Discussion,** entitled "*Conditions for hetero-association and trans-ensemble excitation under the CA3 network of a rat scale*".

Figure 8 — Task design

Strength: clear schematics.

Concern: C3P1 does not test sequence memory; probe failures rescued with cues; only males; no motivation/anxiety controls.

Needed: sequence randomization, removal of within-run rescue, trajectory/latency analyses.

> We tried a sequence randomization in the C1P3 scheme as you suggested, but mice were not able to learn this task at all. Indeed, as shown in Fig 9Da, it took five days for wild-type mice to reach a learning criterion even for a single sequence. Along this line, we had to rescue the probe failure with cues in order not to confuse the mice under training.

For maintaining motivation without affecting general performance, we were very careful for restriction of water to the mice while maintaining their body weight at 80% of normal value (Line 818).

We confirmed that the anxiety level of *Kcna2*+/- mice is normal using an elevated plus maze in our previous study (Figure 3e of Eom et al. 2022)[5].

Figure 9 — Behavior results

Strength: WT vs mutants diverge under C1P3.

Concern: C3P1 not diagnostic; small/uneven Ns; binary data not analyzed with mixed-effects models.

Needed: logistic mixed-effects analysis, effect sizes, behavioral controls, and acute CA3 manipulations or Kv1.2 rescue to establish necessity.

> As we discussed below in response to '*Additional recommendation*', we think that acute overexpression or knock-down experiments are not appropriate for testing the role of LTP-IE in the spatial sequence learning task.

In addition, we re-analyzed our results using a linear mixed effect (LME) model throughout our article (Figures 1 to 5 and Figure 9, and Supplementary Figures 1 to 3). We described statistic parameters such as effect sizes (generally, F-value) and p-value throughout our article.

Minor issues

1) Spell out "CFC" and provide freezing curves.

> CFC stands for contextual fear conditioning. For experiments of Figures 1 to 5, however, we have omitted the habituation step, which is the first step for the standard CFC protocol on Day 1. As mentioned above, in the present study, a single electrical shock was given to ensure that mice turn an attention to the context, not to train animals for CFC. Therefore, we did not use the term, CFC, any more in the revised Ms. Instead, we used exposure to a novel context with a single electrical shock instead of CFC.

2) Report viral titers and infection spread.

> We reported viral titer in line 690. Please note that viral infection was used only in Figure 1 for comparing excitability of ensemble and non-ensemble cells in rats. In other figures we used transgenic mice (cfos-shEGFP mice). In Figure 1, we always performed patch recordings within one or two slices from the focus of viral injection. Because infected cells do not constitutively express fluorescent proteins, we could not routinely check the infection spread. The range of infection spread would be critical for analyzing animal behaviors. But it was not our purpose of the experiments in Figure 1.

3) Clarify animal-level Ns and apply hierarchical statistics.

> We re-analyzed our data using the linear mixed effect (LME) model to count in the variance between experimental animals and slices. The main statistical parameters are presented in the attached Supplementary Data File in a Microsoft Excel format.

4) Replicate key findings under intact inhibition.

> Previously, we have confirmed that MF conditioning can induce potentiation of PP-EPSP in the absence of picrotoxin (Figure 4B of Hyun et al., 2015)[2]. This figure is replicated in our answer to 'Conceptual issues' #2.

5) State sex of animals consistently.

> Only male animals were used in the present study. We explicitly described it in Materials and Methods.

6) Model connectivity should reflect biological values or provide sensitivity testing.

> As aforementioned, our simulation was done fairly under parsimonious conditions compared to real CA3 network of a rat scale. Because it was not practical to simulate sequence learning on the CA3 network of a real rat scale, we estimated what proportion of CA3 ensemble cells is needed to undergo LTP-IE in order for the ensemble to be hetero-associated with the next ensemble in the CA3 network of a rat scale. We found that LTP-IE in about 10% of ensemble cells is sufficient for hetero-association of two CA3 neuronal ensembles representing temporally adjacent two events. Please see ***Supplementary Discussion***, entitled "*Conditions for hetero-association and trans-ensemble excitation under the CA3 network of a rat scale*".

Additional recommendations

1. The Discussion should include a Limitations and Weaknesses section, explicitly acknowledging the aversive conditioning context, possible synaptic contributions, illustrative modeling, and behavioral strategy alternatives.

> We added "*Limitations and weaknesses of the present study*" to Discussion. In addition, please see our replies to your comments in 'Limitation and Weakness' below.

Crucially, the study lacks causal manipulations:

A gain-of-function experiment (e.g., artificially boosting CA3 excitability plasticity and testing sequence behavior) would establish sufficiency.

A loss-of-function experiment (acute CA3 silencing or Kv1.2 rescue/knockdown during behavior) would test necessity. These are essential to establish a causal role for CA3 IE in sequence learning.

> The neuronal excitability is a key regulator for recruiting principal cells to a memory trace. For example, if the excitability is artificially increased in a subset of principal cells using over-expression of CREB or

dominant negative KCNQ2 or hM3Dq DREADD, they were preferentially allocated to memory trace or to an engram encoding fear memory, while principal cells overexpressing Kir2.1 were preferentially excluded from memory engrams [10, 11]. Furthermore, the effects of CREB overexpression was abolished by co-overexpression of Kir2.1 [11]. Artificial manipulation of neuronal excitability using gain or loss of function mutation of ion channels in a subset of principal cells has been used for elucidating the key factors that determine which neurons will be recruited to an engram that encodes a simple association memory [12]. However, the same method cannot be applied to our behavioral paradigm in which multiple neuronal ensembles may have to be timely recruited to encode sequence of multiple memories, because uncontrolled silencing or overexpression of *Kcna2* in a subset of principal cells using viral vectors will preferentially recruit or exclude infected cells to memory ensembles. Therefore we cannot test the essential role of LTP-IE in sequential learning using viral knockdown or overexpression of Kv1.2.

Different from viral manipulation of cell excitability, hetero-knock out (KO) of *Kcna2* causes little heterogeneity in the baseline excitability among principal cells and specifically abolishes LTP-IE in CA3 pyramidal cells. For example, *Kcna2* hetero-KO mice were normal in one-trial contextual fear conditioning and pre-exposure fear contextual fear conditioning, implying intact CA3 recurrent network supporting pattern completion-based recall [5]. These features of *Kcna2*+/- mice provide us a precious opportunity which allows us to investigate the specific behavioral consequences to the lack of LTP-IE with minimal effects on neuronal excitability and without causing population heterogeneity in the excitability, which may result from uncontrolled overexpression or knock-down of Kcna2. We therefore respectfully ask that the requirement to perform corresponding additional experiments be waived.

Limitation and Weakness

1) aversive conditioning context

> As aforementioned, we repeated the experiments of Fig. 1 without a shock in context A. We found that the CA3 ensemble size, the baseline excitability of CA3-PCs, and their LTP-IE inducibility are not different whether the animals experienced a shock or not in context A [**Fig. S1C-D** and associated text, Line 156].

2) possible synaptic contribution to LTP-IE

> We have previously ruled out the possible contribution of NMDAr to MF-induced potentiation of PP-EPSPs (Figure 4D of Hyun et al., 2015, replicated as the second figure in our reply to '*Conceptual issues*' #2).

3) illustrative modeling

> We added Figure 10 to the revised manuscript.

4) behavioral strategy alternatives

> As Review #3 suggested in his/her Major issue #1, we tested wild-type and Kcna2+/- mice for trace fear conditioning (tFC), another temporal associative learning task. Unfortunately, we failed to find any difference in the performance of tFC between these two genotypes. Please see our reply to the comment of Reviewer #3 for details.

Conclusion and recommendation

This is an innovative and ambitious manuscript, but several conceptual, methodological, and interpretational issues need to be addressed. The physiology is intriguing, the modeling creative, and the behavioral design promising, yet the manuscript currently over-claims and omits critical controls.

Recommendation: Major revision. Addressing the aversive conditioning confound, adding synaptic assays, improving behavioral analysis, clarifying reporting, and including causal gain- and loss-of-function experiments will be necessary to substantiate the central claims.

In closing

This manuscript reflects substantial effort and creativity. With additional rigor, transparency, and acknowledgment of limitations, it has the potential to become a field-shaping contribution. I look forward to a strengthened revision.

Thanks,

Reviewer #2 (Remarks to the Author):

The authors of this manuscript use electrophysiology, modeling and behavioral approaches with the goal to understand how intrinsic plasticity determined by changes in IKD in CA3 neurons might contribute to sequence learning. The results might be potentially interesting; however I find that the manuscript is very difficult to read, especially in its first part, because of the way the findings are currently presented.

Some of the issues are detailed below.

1)The numbers for all the experimental (sub)groups presented should be included to be able to better evaluate the validity of the findings in function of the sample size.

> We added the number of cells, slices, and animals for all experiments.

2) It would be useful to have a summary figure to explain the framework, including the synaptic inputs and the kind of plasticity they undergo as they pertain to the various experiments, especially for researchers who are not deeply familiar with the specific topic.

> We added a summary cartoon as Figure 10.

3) The authors should clearly state their definition of CA3 ensemble cells in the beginning, as the term is mentioned but not defined in the introduction (line 93). I assume that it means CA3 neurons that are activated in a specific context, but this in not clearly stated.

> We added following statement in Introduction: “A memory ensemble is a subset of principal cells that are co-activated while a memory is represented in a network.” (Line 94)

4) Some of the names of the experimental subgroups appear to be convoluted, making it difficult to follow the presentation of the findings (see for example ctxAB+, ctxAB++ as well ctxAB(++/1) and ctxAB(++/2) also mentioned below).

> We apologize for the confusion. In the revised Ms, we made efforts for clearer description for ctxAB(+) and ctxAB(++), which represents cell subgroups displaying low and high EGFP expression (Fig. 3Be). CtxAB(++) were further separated into two subgroups depending on their baseline input conductance (G_{in}) (Fig. 3Cc). Please see line 228 (underlined) and line 231 (underlined).

5) In Fig. 3, were ctxAB(++/1) and ctxAB(++/2) cells recorded from the same or different animals?

> Both category cells were found in the same animals.

Minor:

Abstract lines 41-42: something is missing in this sentence.

> We rephrased this sentence as “Here we show that CA3 ensemble cells activated by visiting a novel context display high excitability, while the excitability states of those activated by re-visiting a similar context diverge into high and normal excitability states.”. Please see Line 42.

Introduction, lines 59-63: reference should be added for these statements.

> We cited a following book chapter in the text: “Amaral D, Lavenex P. Hippocampal neuroanatomy. The hippocampus book. New York, NY, US: Oxford University Press; 2007. p. 37-114.” (Line 61, Reference #6)

Results: is there a reason why the experiments in Fig. 1 are performed in rats whereas the rest are in mice?

> Previously our *in vitro* slice electrophysiology experiments were mainly performed in rats, and thus we first confirmed LTP-IE *in vivo* in the rats. However, we need to repeat it in mice because CA3-Kcan2+/- rats, which lack LTP-IE, were not available for behavioral tests.

Results, lines 145-148: it is unclear how these sentences refer to Fig. 2, since only fluorescence data is presented in this Figure.

> We are sorry for this confusion. However, we think this introductory remark necessary for readers to understand why we need to do the experiment shown in Figure 2. To highlight our purpose for the experiments in Figure 2, we re-phrased the introductory statement (Line 175).

Results, line 134: is ctxAB(++) here a typo? Because the two context experiments are not introduced until later.

> Yes, it is. We apologize our mistake. We deleted it.

Results, Figure 3Be: the term shEGFP(++) is not introduced anywhere in the manuscript.

> We defined shEGFP(++) in Line 231 (underlined).

Results, lines 236-238: the sentence is unclear.

> We corrected this sentence as "*Because ctxAB(++/1) and ctxAB(++/2) cell groups exactly overlapped with the ctxAB(++) cell subsets displaying high and low EPSP/EPSC ratio, respectively, ctxAB(++) cells exhibiting high EPSP/EPSC ratio could be regarded as ctxAB(++/1) cell, and the other subset as ctxAB(++/2) cell (Fig. 4Ab).*". Please see line 280 (underlined)

Methods, line 538: the sentence about glutathione is unclear, since it is not mentioned elsewhere.

> We deleted the sentence because we have already mentioned the composition of internal solution in the same paragraph.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors tested their hypothesis that Kv1.2 channels in CA3 pyramidal cells contribute to sequence learning. The group focuses on the role of Kv1.2 channels in cellular excitability for years. In this manuscript they expand their previous in vitro work to test their hypothesis in behaving mice and a computational model. They first demonstrate that exposure of mice to a novel context increases cellular excitability in a Kv1.1 and Kv1.2 channel dependent manner. Second, authors propose a computational model where this mechanism can form a sequential synaptic link. Third, they show that mice with knockdown of these channels are impaired in a sequence memory task. The combination of these results is novel and interesting, addressing the behavioral relevance to the potassium channel dependent plasticity of intrinsic excitability. These studies that aim to understand the role of molecular level mechanism in cognition have potentials to develop future treatments for cognitive deficits. However, there are several major issues as I describe below.

Major:

1. The behavioral task that the authors used does not support strongly the mechanism the authors are interested in is involved. This is because there is no temporal discontinuity in this task. Firing of place cells in CA3 that fire at different locations on the maze are known to overlap in time as the animals run. They can therefore be linked through a simple STDP mechanism without involving IKD mechanism that the authors focus on this paper. Better tasks are, for example, trace conditioning or sequence of odor presentations separated by temporal gaps, which have been shown to depend on hippocampus.

> Thank you for these thoughtful suggestions. We tried inter-trial intervals longer than 5 s (10 or 15 s), but could not get a learning curve even in the WT mice. Lengthening the inter-trial interval disrupted the task performance, probably because mice failed to maintain their focus on the task goal during the long interval. We think that mice might not be sufficiently attentive to the spatial sequence learning task when longer intervals were used. We have also attempted to test the Eichenbaum-style odor sequence learning task in mice, but it was not successful even in the wild-type mice, probably from the same reason why mice failed to learn the spatial learning tasks with an inter-trial interval of 10 s or longer. From the same reason, most sequence learning tasks might have been tested in rats, not in mice [13] [14] [15]. Because *Kcna2* KO rats were not available, we had to test mice for the spatial sequence learning task.

Please note that inter-trial intervals were set to 5 s. During encoding phase of the training, mice should remain idle for 5 s until next target cue is presented. Given the time constant of spike-timing-dependent potentiation (STDP) is less than 100 ms, the 5 s interval is long sufficient for prevent synaptic LTP through NMDAr-dependent STDP. Although two adjacent memories can be recalled in a very short time once an animal has learnt a sequence, it is not the case while animals encode a novel sequence.

As Reviewer suggested, we have also tested the possibility for LTP-IE to contribute to trace fear conditioning (tFC) (**Fig. R3**). However, CA3-*Kcna2*+/- mice were normal in the tFC task, consistent with

Suh and Tonegawa (2011)[16]. It needs further study why CA3 does not contribute to tFC.

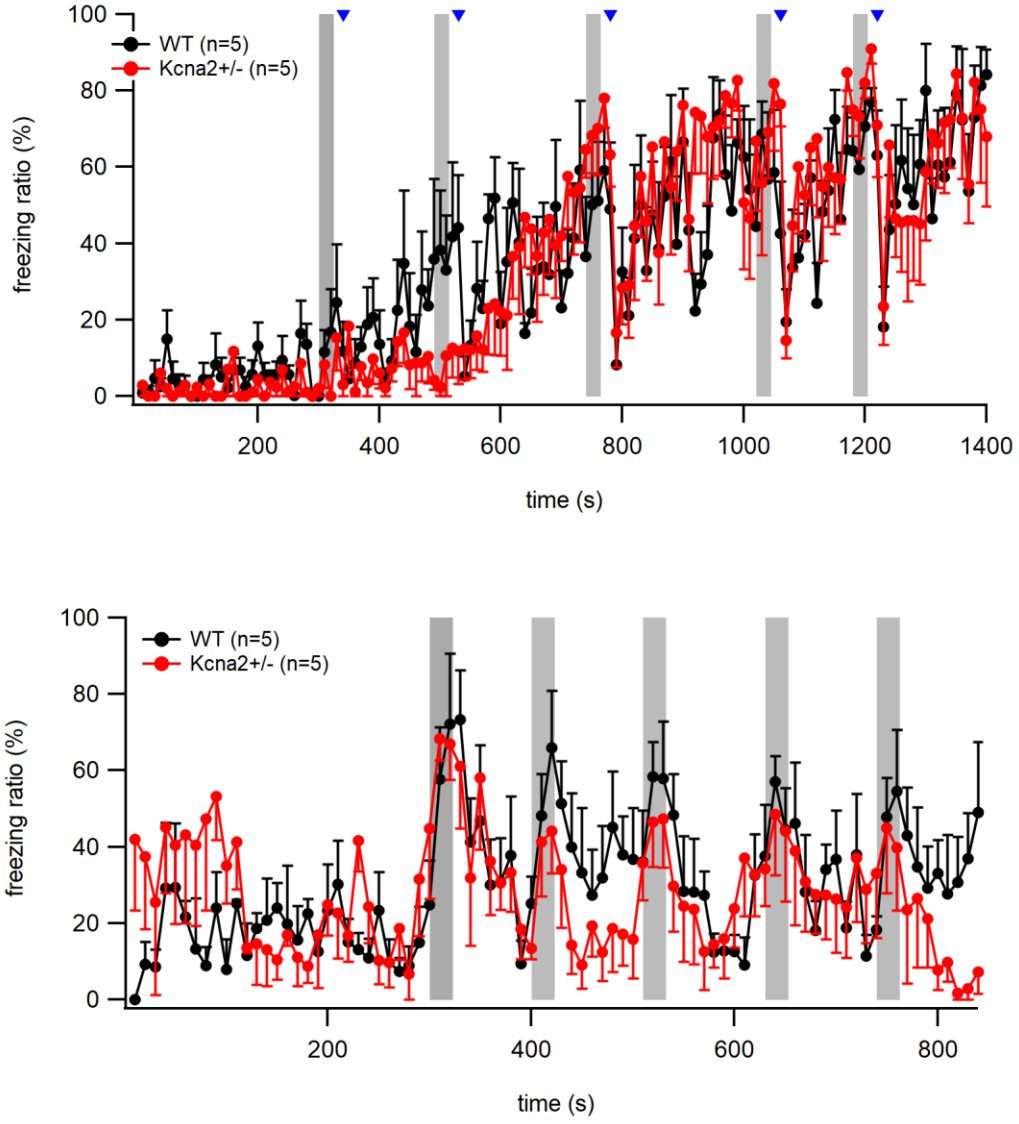


Figure R3, Time courses of freezing ratio during TFC (*upper*, Day1) and tone test (*lower*, Day2) on the next day in Kcna2+/- mice (red) and their wildtype littermates (black). Time periods for presentations of 20 s tone were indicated as shaded boxes. On day 1, 0.5 mA shock was given for 2 s after the end of each tone (*triangles*). On the next day, responses to the same tone of the mice were tested in a distinct context from that of day 1. Freezing ratio was measured every 10 s as a proportion of motionless duration. The methods employed for trace fear conditioning is described in detail in Lee et al. (2004)[17].

Methods for Trace Fear conditioning: The fear conditioning was performed in a context A, which is a mice conditioning cage (18 cm wide × 18 cm long × 28 cm high; H10-11M-TC, Coulbourn Instruments, USA) located inside a sound-attenuating box (SCITECH, Korea) with an LED lamp. An auditory cue was presented via a speaker module (H12-01R, Coulbourn Instruments, USA) located on one side of the

conditioning chamber. The floor of the conditioning cage consisted of stainless-steel bars to deliver a footshock through an electrical stimulator (H13-15-220, Coulbourn Instruments). Context B is rectangular (16 cm wide × 16 cm long × 20 cm high) and consists of a white acrylic floor, and a white acrylic wall with a black check pattern. The experimental protocol was performed by Ethovision XT 13 software. To quantify freezing behaviors, a python-based open source code ezTrack (Pennington, 2019) was used. The parameters we used for this analysis were a motion cutoff of 20, a freezing threshold of 30, and a minimum freeze duration of 30 samples. The behavior was recorded with a CCD camera.
CA3-Kcna2 +/- mice and wild-type mice were used for trace fear conditioning experiments. In trace fear conditioning (day 1), the mice received five CS-US pairings which were delivered at 300, 490, 740, 1020, 1180 sec. The CS-US pairing consisted of tones (2.5kHz, 20 s, 75 dB) and a footshock (0.5 mA, 2 s). CS and US are separated by a 20 s trace interval. The intertrial intervals were 220 ± 60 s. After each test, the context A was cleaned with 70% alcohol. The next day, in the tone test (day 2), the mice received five presentations of tones (20 s, 2.5 kHz, 75 dB) which were delivered at 300, 400, 510, 630, 740 sec in context B. The intertrial intervals of tone presentations were 110 ± 10 s. After each test, the chamber was cleaned with 1% ammonia.

2. Grouping of cells and statistical analysis in ex vivo experiments are not scientifically appropriate in Fig1-4.

2-1. In Fig1, to address the effect of cell activation, which is marked by the EGFP expression, Gin of all EGFP- and EGFP+ has to be compared. It is not scientifically correct to separate observed results into groups based on observed measurements (analysis is circular). The interpretation that "EGFP(-/low) cells may represent memory ensemble cells that are not infected by AAV" might be true. But it is not scientific to separate observed data points into groups based on such speculation. Authors need evidence.

> We appreciate the reviewer's concern that separating EGFP(-) cells into high and low G_{in} groups based on observed measurements may constitute circular reasoning. To resolve this, we have employed silhouette analysis[18], a gold-standard unsupervised clustering validation method, to objectively determine whether the EGFP(-) population contains distinct subgroups. Based on this concept, we re-wrote the text describing Figure 1B. Please see line 125-135.

2-2. Similarly, in Fig 3, the interpretation "Because cells with distinctly high G/R ratio were observed only in the mice which visited both context A and B, the ctxAB(++) cells may be activated in both contexts, while cells in the other group were activated once." is a speculation. It is not correct to separate the results into two groups based on the value you observed from ctxAB group based on such speculation. It is obvious if one separates observed group into high and low data points that you will get significant difference between those groups (which is shown in Fig3 Be ctxAB+ vs ctxAB++ comparison with *** $p<0.0001$). Similarly, if you take the high value group of ctxAB group and compared to the ctxA+ group, finding out that ctxAB++ group is higher (which is shown in Fig3Be as well) is nothing to do with the fact that the animal went through ctx A and B, it could just be because authors took high values from ctxAB group.

2-3. In Fig3 C to E, it is necessary to have the **ctxAB group** which include all ctxAB+, ctxAB++1 and ctxAB++2 groups together. If further sub-categorization is needed, authors can add extra analysis where the authors

focus on sub grouping of ctxAB group with careful justification.

> For comments #2-2 and #2-3, we acknowledge the reviewer's concern that categorizing G/R ratios of ctxAB cells could appear as speculation. Following the same rigorous validation of clustering as in Figure 1, we applied silhouette analysis for k-means clustering to objectively determine the optimal clustering of ctxAB cells. Please see line 226-229 and Fig. S3.

3. In Fig2, how do we know that this difference of fluorescence intensity distribution is not simply due to the time from the last exposure to a context? To address this, context B only exposure followed by ex vivo experiment 60 min later is needed.

> We have already shown that fluorescence intensities of EGFP(+) cells are not different at 60 and 90 min after single exposure to a novel context (context A) in Figure 1B (red and blue circles) and in Figure 3Be [ctxA60(+) vs. ctxA90(+)]. Consistent with this result, Figure 3 in Chowdhury & Caroni (2018; www.doi.org/10.1038/s41467-018-06516-3) have shown that the fraction of cFos(+) cells remains unchanged until 180 min in the hippocampal CA3 area [19]. Therefore, it is unlikely that the time from the last exposure to a context caused the difference of fluorescence intensity distribution between ctxA and ctxAB mice.

4. In the modeling study, authors used PP input to all groups (M1-M4). This is misleading considering the fact that the authors stress "orthogonal" memory representation. Since the model heavily rely on this, it should be justified based on empirical data.

> We added theoretical plausibility that all the CA3 principal cells may receive synaptic inputs from ensemble cells in the entorhinal cortex (EC) via perforant pathway (PP) in **Supplementary Discussion** (subtitled "*Plausibility for all the CA3 principal cells to receive ensemble inputs from EC via PP* "). Assuming random connections between EC and CA3 principal cells, this calculation predicts that most CA3-PCs receive synaptic inputs from more than 300 ensemble cells of the EC. Therefore, all CA3-PCs have a potential to be re-activated by PP during memory retrieval as long as the PP synaptic weight is sufficiently high. For a CA3-PC to have high PP synaptic weights, a help of concomitant MF inputs is essential because MF inputs provide depolarization required for inducing PP-LTP (homosynaptic LTP at PP synapses)[7]. Moreover, the present and our previous studies showed that, if the MF inputs are sufficiently strong, the postsynaptic CA3-PC undergo LTP-IE, and can be also recruited for encoding of the next event at PP synapses. Therefore, the recruitment of a CA3-PC to a PP synaptic ensemble is dictated by sparse MF inputs. Along this line, MF inputs during an encoding phase is essential for a CA3 network to perform pattern separation. Once the synaptic weights at PP and A/C synapses are modified with a help of MF input, however, memory ensembles encoding similar memories can be distinctly retrieved by PP inputs alone, as predicted by Treves and Rolls (1994)[20] and evidenced by Lee and Kesner (2004)[21].

4. Different mechanism that are proposed to support temporally separated events should be mentioned in the introduction and discussion (e.g. persistent activity, replay, short-term synaptic plasticity). In

addition, other mechanisms involved in activity induced change in excitability should also be mentioned (e.g. H-current).

> Thank you for this suggestion. We added two paragraphs to Discussion section entitled "*Activity-dependent regulation of ion channels in the CA3 area*" and "*Other mechanisms for hetero-association of temporally separated events*". Please see Line 553 and 598.

Minor:

It should be written in the abstract and introduction that labeling of active cells in context exposure was done in this study.

In the abstract, the statement that "Here we show that the high excitability of CA3 ensemble cells in the animals experienced an event can be normalized by a subsequent event." is not supported. This, as the authors discuss in the Discussion, is an interpretation.

> We rephrased the statement as "Here we show that CA3 ensemble cells activated by visiting a novel context display high excitability, while the excitability states of those activated by re-visiting a similar context diverge into high and normal excitability states."

L 52: English needs to be checked by a native speaker. For example, "capture hypothesis may link two neuronal ensembles" is probably wrong because "hypothesis" does not "link" memories.

> We apologize for the confusion. We meant "*synapse tagging and capture hypothesis"* that was first proposed by Frey and Morris (1997)[22] and has been supported by subsequent papers [reviewed in Rogerson et al (2014)] [23]. To avoid confusion, we italicized the phrase "*synapse tagging and capture hypothesis"* in the text.

L 118: "Whole-cell patch" -> "Whole-cell patch recording"?

> We corrected it to "Formation of the whole-cell patch configuration" (Line 123).

L 134: ctxAB(++) appears first time here and it is not clear what it means and how this is relevant.

> We apologize our mistake. The "in ctxAB(++) cells" was erroneously inserted, and thus we deleted it.

Fig1.B: It should be indicated from which group each EGFP- cells came from. Did the EGFP(-/low) group came from specific group (60, 90 or 180 min)?

> We explicitly described that all GFP(-) cells were obtained from animals of 60 min. Please see the legend of Fig. 1B.

Fig1.Cc: Is it a mistake that EGFP(-/high) shows n.s.?

> The horizontal range bar was erroneously drawn. Thank you for pointing this out. We corrected it in Fig. 1Cc.

Fig1.B: If the authors' main claim is that inclusion of cells into assembly causes the reduction of Gin, then they should show statistical comparison of the 60 and 90 min group compared to control and EGFP- groups (without separating them into low and high).

> Complying with your suggestion, we added a statement for comparison of the 60 and 90 min group with the control and EGFP(-) groups (Line 125). As you expected, we found no difference when EGFP(-) cells were not separated. Nevertheless, given that the infection rate is about 50% (Fig. S1B) and that there was no EGFP(-) displaying low G_{in} in the c*fos*-shEGFP-transgenic mice (Fig. 3Cf), it is likely that the EGFP(-/low) cells may represent memory ensemble cells that are not infected by AAV (Line 142).

L 216: underwent -> undergone

> Thank you. We corrected it as you suggested.

L 261: It helps if there is a summary statement of this section at the end.

> As you suggested, we added a summary statement (Line 308).

Fig. 6 and 7: The text goes back and forth between Fig6 and 7. Modify the figures or the text to avoid back and forth.

> Figure 6 and associated text were corrected as you suggested.

L 344: The explanation of the task is hard to understand. Especially, it is not clear enough for the first time reader how the sequence is implemented. Instead of "A 'run' was completed, when a mouse visited four towers in a sequence (Fig. 8A).", it would be better to write something like: "A sequence animal needed to learn was defined by a 'run' which consisted of four trials (i.e. visiting of four towers) ".

> We appreciate your kind suggestion. We corrected the statement as you suggested (Line 410).

L 348: "guided run" should be defined.

> Guided run is defined as a run in which all trials are guided by light cue. We defined in the text (Line 405).

L 390: This possibility that some ctxAB cells that went back to normal excitability level is a natural flow of the paper which is expected to be mentioned in the results section than the discussion section.

> Thank you for your suggestion. Complying with it, we added a summary paragraph (Line 308).

L417: “are lacking”

> We corrected it as you suggested.

L423: “little has been understood”

> We corrected it as you suggested.

L425: Logic around here is difficult to follow. Especially, it is often unclear if the word “overlap” is used for temporal overlap or spatial (neuronal representation) overlap . This is the case also in Line 36 “non-overlapping representation of memories”.

> We meant an overlap between ensembles representing different memories. In Abstract, we used more formal word, ‘orthogonal’, instead of ‘non-overlapping’. We explicitly defined ‘ensemble overlap’ in the context of the paragraph as “ensembles representing temporally separated episodes to share a number of principal cells (called ensemble overlap)” (Line 473).

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Reviewers' Comments (2nd Round)

Reviewer #1 (Remarks to the Author):

Dear authors,

The revised version of the manuscript has answered my concerns to a large extent. I have no further comments. Congratulations on your interesting study.

Best Regards,

Reviewer #2 (Remarks to the Author):

The authors addressed most of the issues I raised in my prior review; however, there are still a couple of issues.

MAJOR

Figure 10 does not clarify and, if possible, makes the summary more confusing.

What I was asking was to identify which inputs (MF, vs PP vs A/C) are causing what type of plasticity and in which order. This is missing in the diagram. In addition, only event 1 and 2 of the four events in the diagram are described in the figure legend. Finally, at line 1039, does non-MF inputs refer to A/C input, PP inputs or both?

> We appreciate this constructive comment. Complying with your suggestion, we thoroughly revised the Fig. 10 to append external synaptic inputs to the model network and consequent modification of recurrent synapses. In addition, we re-wrote the figure legend to cover all events (Event 1~4) and thus to explain whole processes of sequential memory formation in the CA3 network.

> The non-MF inputs refer to PP inputs in the line 1309 (line 1039 may be a typo of line 1309). We replaced "non-MF inputs" with "PP inputs" in the revised Ms.

MINOR

Fig. 2 does not report the number of samples in this data set. In all other data sets, the number of samples is reported in the figure legends, except for Fig. 5 where they are reported in the text. This seems an odd choice; for consistency, it may be better to report them all in the figure legends.

> The sample size of the dataset in Fig. 2 was added to the figure legend as (number of cells/number of

slices/number of animals) in the same way as in other figures.

> The number of datasets for Fig. 5 has been deleted from the text, and moved to the figure legend.

Reviewer #3 (Remarks to the Author):

The first major concern I raised in the initial review remains: there is still a substantial gap between the behaviour task and the remaining. Increased excitability in the in vitro study was examined in relation to a novel context and aversive stimulation, neither of which is relevant in the 5-arm maze task. While the 5-arm maze task involves a sequence, the in vitro work used a novel context as the induction for IKD dependent mechanisms; therefore, it does not provide evidence that increased excitability occurs when the mice visited each arm or when the light cue was turned on. My previous mention of trace fear conditioning was to potentially address this exact point. The authors have, however, indicated that trace fear conditioning was tested but was unaffected.

> We agree with the Reviewer's concern that there is a large gap between our *ex vivo* and behavioral studies. However, it was not feasible to test the excitability changes of CA3-PCs during the five-arm maze. The exposure to a novel context with a foot-shock was employed in the present study, because this was a simple and practical way to test excitability changes of CA3-PCs *ex vivo*. Although we did not directly test excitability changes during the five-arm maze task, salient events in our five-arm maze task, such as reward delivery and the light cue, may engage hippocampal plasticity-related processes in light of a previous study in the CA1[3]. We agree, however, that this remains indirect, and we have revised the text to clarify this limitation.

In the revision, the authors pointed out that the change in increased excitability was not mainly due to the footshock, but rather the new context. I find it difficult to reconcile the precisely timed input in the model (which induced increased excitability) with context exposure. As far as we currently know, context exposure lacks such precisely timed, distinct inputs. Such time-constrained stimuli are more consistent with a footshock. This, unfortunately, represents a new mismatch that I noticed in this revision.

> We imagine that not only temporally discrete stimuli but also contextual inputs may induce an the excitability change in CA3-PCs as long as they induce MF-induced burst firing in CA3-PCs in light of our previous *in vitro* studies. It remains to be tested, however, whether visiting a reward zone (a target tower) in the five-arm maze can induce LTP-IE in CA3-PCs as we assumed in the simulation. To confirm this view, we may need *in vivo* patch-clamp study on CA3-PCs in freely moving animals. Because it is not trivial, this question should be addressed as a further study in the future.

The authors claimed that the 5-second temporal gap between trials provides a discontinuity that requires mechanisms longer-lasting than NMDA-dependent synaptic plasticity. However, studies indicate that place cells remain active during immobility (Hok et al, Journal of Neuroscience, 27 (3) 472-482, 2007). It has also

been shown that “time cells” can bridge such temporal gaps of up to 5–10 seconds (Pastalkova et al., Science, 321(5894):1322-7, 2008). These indicates that hippocampal activity could bridge the 5-second gap. Therefore, the situation is unlike the simulation, where there is no input or firing in the hippocampus for 5 seconds. If this activity maintains the information about the current arm, the association between the current arm and the next light cue might be done with NMDA-dependent time scale. In fact, it has been reported that place cells and time cells can fire differently depending on past trajectory of the animal (Wood et al., Neuron 2000 27(3):623-33 or Pastalkova et al., Science, 321(5894):1322-7, 2008). This is highly relevant to the manuscript because sequence execution requires maintained history (which arm the mouse visited for far).

These studies do not exclude the possibility that IKD based mechanism is involved. However, place cell firing is well described in radial mazes, and they are thought to be implicated in the formation of sequence (e.g. through “phase precession” and “theta sequence”). If it is not possible to change the behavior study to better align with in vitro part, the authors could at least add or modify the simulation part to better integrate the 5-arm maze behavioral result by incorporating well described place cell and potentially time-cell activity in the model together with IKD-dependent dynamics.

> We are grateful for this insightful comment. As Reviewer suggested, we examined how place cell firings during inter-event intervals influence on encoding of sequence events in our network model. To simulate encoding of a sequential events in the presence of place cells firing, each inter-event interval was intervened by eight place fields, and each place field was represented by firing of 40 CA3-PCs, which is one-fifth of an M subset in size. The simulation results described in the text (line 438) associated with Figure S4, and discussed the possibility for the role of time and place cells in hetero-association of episodic memories (line 573). As newly described in Discussion, recent studies revealed that animals and humans replay a sequence of multiple episodes during or after non-spatial tasks requiring memory of event sequence [1] [2]. It remains to test whether our synaptic mechanism may underlie such replay of non-spatial tasks.

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Reviewers' Comments (Final round)

Reviewer #2 (Remarks to the Author):

The authors addressed all my prior concerns.

Reviewer #3 (Remarks to the Author):

Dear authors,

My concerns were all addressed by this round of revision. I hope the authors advance this field by conducting in vivo recordings in behaving animals in the future.