nature portfolio

Peer Review File

The massed-spaced learning effect in non-neural human cells

Corresponding Author: Professor Thomas Carew

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Kukushin and colleagues present very creative work to clarify and demystify cellular mechanisms that underlie the difference between spaced and massed training on long-term memory. They demonstrate that non-neural SHS5Y cells show clear differences in long-lasting CREB-dependent gene expression induced in response to identical levels of nett stimulation, delivered differently, either in “massed” fashion, or “spaced” fashion. The authors use combination of forskolin and TPA, to activates the PKA pathway and PKC pathways respectively, selected to mimic the effect of 5HT receptor activation in Aplysia sensorimotor synapses, where massed vs spaced effects have been well analysed by the authors previously. This work therefore establishes a simple and accessible cellular preparation in which the mechanisms underlying the massed/spaced difference that can be deciphered far more easily than in nervous systems in vivo. Overall, the problem being addressed, the quality of the daya, and progress are impressive and merit publication.

I do however have suggestions for issues that need to be better clarified in the text to potentially provide intellectual depth and improved perspective to the reader. In general, it appears that: (1) past relevant work and their implications could be discussed (and credited?) more than currently done, (2) prior work and models on how the duration of ERK signalling could be interpreted by downstream mechanisms neurons and other cells would be worth mention and citation, and (3) some potential differences between the details of the effects seen in the nervous system vs SHS5Y cells should be formally acknowledged (or the similarities more explicitly clarified). These additional sections are probably more relevant and useful than some of the more general background in the introduction and discussion.

1. The uniqueness of how temporal nature of signals are interpreted in non-neuronal cells seems somewhat overstated in the Introduction, and I think a more measured and scholarly tone would be more appropriate. For instance there have been a wealth of studies on how the duration of MAPK activation could be controlled in cells: in particular on how “bistable switch” like behaviors for ERK could emerge. These include experimental and computational work by by Jim Ferrell and colleagues many years ago, as well as computational work by Ravi Allada (though I am unsure if these includes PKA and PKC). I suspect that it would be relatively easy to build timing into prior models (if this hasn’t already been done) based on the relative efficiency and kinetics of MAPK phosphatases and MAPK kinases.

2. There have been prior models for how different effects of massed vs spaced stimulation and transient vs sustained ERK signalling could lead to different forms of gene expression. For instance, there is the attractive idea that a long-lasting ERK signal could not only activate CREB but also down-stream immediate early transcription factors. https://www.nature.com/articles/ncb822 a mechanisms that could be relevant across multiple cell types and signaling pathways. I think the reader will gain a lot from being provided a fuller perspective on past/ contemporary models, not least to appreciate what precisely what progress is represented and/or enabled by the discoveries described here.

3. The authors find the CRE-Luc-PEST activity remain high 20 hours after spaced stimulation. Is there any evidence that IEGs are transcribed for so long after training in neurons? My understanding is that while LTM and related plasticity can be seen after such long time scales, the direct targets of CREB a(IEGs) are transcribed for much briefer, early time intervals. This should be carefully specified. I stress that it remains very interesting that the fundamental massed/spaced stimulation effect on gene expression is so beautifully reproduced in these cells. But it is important to acknowledge potential differences across cells types (which may even exist across neuronal types). I suggest that this be done in a discussion paragraph where the value of this work for future discovery is highlighted.

4. Please include a short discussion paragraph where the value of this work for future discovery is highlighted.

5. I expect that the older work indicating a role for degradation of the PKA regulatory subunit and sustained PKA activation is

“upstream” of the processes being investigated here). Still maybe worth a mention.

6. Page 3 – minor typo “cellular kinase may be involved,” (the “be is missing)

7. Page 4 (or later): a brief explanation for why only the first and last of the spaced stimulations matter (and not the intermediate ones) in the Aplysia prep will be useful. Particularly since this does not seem to be the case for SHSY5Y cells.

8. The introduction could be a bit less general and go deeper on a few more sophisticated points (suggested in these comments).

9. Page 7: may be clearer to add “for each” so a sentence in the last para would read” “elicited minimal effect after a single pulse of each”

10. Page 8 last para similarly perhaps change to:” may not have be possible to observe a potential gene expression correlate of “cellular forgetting”.

11. Somewhere please clarify whether Forskolin and TPA can substitute for 5HT in Aplysia. (e.g page 9 end of first para)

12. Nuclear translocation of P-ERK is clearly critical for CREB activation. A little more attention to the set up and discussion of these experiments and data will be useful.

Signed: Mani Ramaswami

Reviewer #2

(Remarks to the Author)
The authors present an alternative model system to study the molecular mechanisms of the massed-spaced effect of memory formation utilizing the immortalized neuroblastoma cell line SH-SY5Y in conjunction with a short lived CRE-luciferase reporter. First, the authors show that a spaced training paradigm, simulated by stimulation with the PKA and PKC activators forskolin and TPA, is conserved in SH-SY5Y cells. Then the authors show that a spaced training also leads to an increased activation of Erk1/2 kinase compared to control or massed training. Together, these findings suggest that the impact of spaced as opposed to mass training occurs at least in part at the level of intracellular signaling pathways.

Major comments

1. It would be helpful for the reader to show a more detailed pathway in Figure 1 depicting the known pathways of how forskolin or TPA lead to ERK and CREB1 activation. Similarly, the authors could provide more information about the known pathways underlying the massed-spaced effect in the introduction in neurons. I would also note that what is missing from Figure 1 is the fact that non-neuronal cells are not islands, but receive signals from other cells, from extracellular matrix, from blood, lymphatics etc. As such, the dichotomy presented feels somewhat simplistic.

2. To further investigate the molecular mechanism of sustained CRE-luc expression, the authors should reduce Erk1/2 function for example by using an inhibitor or knocking down Erk1/2.

3. The labeling of the Western Blot in Figure 5A is confusing. Does it show t=0 for the three different training paradigms? It appears that a label is missing to indicate the specific training paradigm.

4. The authors should explore how increased activation of Erk1/2 leads to sustained CRE-luc expression. Is phosphorylation of CREB1 sustained as well and is this required for the observed effect? How would increased Erk1/2 activity lead to sustained CREB1 phosphorylation? What other kinases/phosphatases are involved?

5. To further generalize their observations, the authors should test if they find sustained CRE-luc expression in cells completely unrelated to neurons, for example in HeLa or HEK293 cells. SH-SY5Y are neuroblastoma cells and serum deprivation is the first part of their differentiation protocol. It would be important to determine if other, completely non-neuronal cells would exhibit the same behavior. Without this type of data, I think it’s not quite accurate to state that “canonical features of memory… can be embedded in the dynamics of signaling cascades conserved across different cell types,” especially since they have only examined a neuroblastoma cell line. Along these lines, isn’t it also known from earlier work, including from the Carew lab (Kukushkin et al PNAS 2022), that spaced applications of 5HT alter signaling in isolated sensory neurons (ie that are not part of a neural circuit)?

6. The discussion is very short and should be elaborated on. What are the potential pathways that lead to persistent CRE-luc expression? What is known about these pathways in neuronal cultures and tissues?

Minor comments
1. The authors did not define most abbreviations used in the manuscript. Please correct.

2. In the paragraph describing Figure 3 the sentence below should be as followed:

The most dramatic differences between the effects of a single pulse and repeated pulses (29% vs 381% compared to

untreated controls after 24 h, respectively) were observed for TPA, which is especially striking since the length of TPA treatment had hardly any effect of luciferase expression (Fig. 2).

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3. It was not clear what vehicle Foskolin and TPA were dissolved in prior to adding to the media, and/or whether this solvent was also used as a control (eg if it was DMSO or ethanol).

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors mimicked training using repeated pulses of forskolin and TPA, and measured CRE-luciferase reporter gene expression at various points. They developed a short-lived reporter system from the human neuroblastoma cell line (SH-SY), stably transfected with a CRE-dependent, PEST-modified luciferase gene. Four spaced pulses of either agonist elicited stronger and more sustained luciferase expression than a massed treatment, demonstrating a form of temporal pattern discrimination. Spaced pulses also resulted in stronger phosphorylation and nuclear translocation of ERK, indicating that the temporal pattern, at least in part, is decoded either by ERK itself or its upstream kinases, suggesting that canonical features of memory do not necessarily depend on neural circuitry, but can be embedded in the dynamics of signaling cascades conserved across non-neuronal cell types. The experimental design and data interpretation are sound and the manuscript was well written. I recommend its publication after improving the following minor points.

1. Citation: A similar massed vs. spaced reporter gene assay was done previously using Aplysia neurons which employed serotonin (5HT) as the memory-inducing stimulus. It could be referred in introduction or discussion.
-Kaang BK, Kandel ER, Grant SG. Neuron 10:427 (1993)
2. The authors argue that their reporter cell line can substitute a neuronal system in some aspects and can be used for the development of predictive models of memory formation. However, their message needs to be tone down. They may also discuss the limitations as the non-neuronal cell system apparently lacks synapses, synaptic plasticity and a cross talk between cell soma and synapse, etc.
3. Typo in line 44: may involved in..

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been comprehensively and carefully revised. I congratulate the authors on a very nice and interesting study!

Reviewer #2

(Remarks to the Author)
This revised application is improved with the inclusion of experiments done in HEK293 cells, inclusion of experiments with MEK inhibitors and experiments examining CREB phosphorylation. It represents a reductionist approach to understanding the impact of spaced versus massed stimulation on memory formation, which, as the authors point out, can be used to guide "formalized molecular models of memory formation."

A couple of comments:

In figure 1, it's not clear if the timing on the y axis of the panel on the left refers to timing of functional impact of the process or to the timing of the process itself. Changes in gene expression, for example, can occur over a time scale of minutes. Regarding my earlier comment about the simplicity of figure 1, I was not referring to the type of simplification of signaling pathways that is now provided in supplemental figure 1 (which I do not think is necessary), but rather to the fact that the synaptic signaling that is considered "neuron-specific" in the right panel has clear parallels in non-neuronal signaling in the form of temporal patterns of extracellular signaling.

It's important to note that both TPA and elevations of cAMP, including by forskolin, differentiate SH-SY5Y cells into neurons (Kovalevich and Langford 2013, Monaghan et al 2008), and as such the vehicle treated control is not quite right as the TPA and forskolin treated cells are undergoing differentiation while the vehicle treated are not.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns adequately.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I support this being published

Dear Reviewers,

We would like you for your time and constructive suggestions. Below, we address your comments point by point.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Kukushkin and colleagues present very creative work to clarify and demystify cellular mechanisms that underlie the difference between spaced and massed training on long-term memory. They demonstrate that non-neural SHS5Y cells show clear differences in long-lasting CREB-dependent gene expression induced in response to identical levels of nett stimulation, delivered differently, either in "massed" fashion, or "spaced" fashion. The authors use combination of forskolin and TPA, to activates the PKA pathway and PKC pathways respectively, selected to mimic the effect of 5HT receptor activation in Aplysia sensorimotor synapses, where massed vs spaced effects have been well analysed by the authors previously. This work therefore establishes a simple and accessible cellular preparation in which the mechanisms underlying the massed/spaced difference that can be deciphered far more easily than in nervous systems in vivo. Overall, the problem being addressed, the quality of the daya, and progress are impressive and merit publication.

Thank you for this favorable assessment.

I do however have suggestions for issues that need to be better clarified in the text to potentially provide intellectual depth and improved perspective to the reader. In general, it appears that: (1) past relevant work and their implications could be discussed (and credited?) more than currently done, (2) prior work and models on how the duration of ERK signalling could be interpreted by downstream mechanisms neurons and other cells would be worth mention and citation, and (3) some potential differences between the details of the effects seen in the nervous system vs SHS5Y cells should be formally acknowledged (or the similarities more explicitly clarified). These additional sections are probably more relevant and useful than some of the more general background in the introduction and discussion.

Thank you for these suggestions. The manuscript now acknowledges prior work more thoroughly, and a discussion of differences between the effects seen in cell culture vs the nervous system is provided.

1. The uniqueness of how temporal nature of signals are interpreted in non-neuronal cells seems somewhat overstated in the Introduction, and I think a more measured and scholarly tone would be more appropriate.

The overall tone of the manuscript has been revised throughout the manuscript. Care has been taken to achieve a more measured tone especially in the Introduction and Discussion, so as not to overstate the implications of our work.

> *For instance there have been a wealth of studies on how the duration of MAPK activation could be controlled in cells: in particular on how "bistable switch" like behaviors for ERK could emerge. These include experimental and computational work by by Jim Ferrell and colleagues many years ago, as well as computational work by Ravi Allada (though I am unsure if these includes PKA and PKC). I suspect that it would be relatively easy to build timing into prior models (if this hasn't already been done) based on the relative efficiency and kinetics of MAPK phosphatases and MAPK kinases.*

We now provide a citation to the Huang-Ferrell model of ERK signaling. However, we must emphasize that the current manuscript primarily aims to demonstrate the utility of our reporter system, rather than provide a complete account of the massed-spaced effect. We are currently investigating the specific role of ERK double phosphorylation in the massed-spaced effect, and the molecular modeling to which the Reviewer refers will be discussed extensively in this follow-up study.

> *2. There have been prior models for how different effects of massed vs spaced stimulation and transient vs sustained ERK signalling could lead to different forms of gene expression. For instance, there is the attractive idea that a long-lasting ERK signal could not only activate CREB but also down-stream immediate early transcription factors. https://www.nature.com/articles/ncb822 a mechanisms that could be relevant across multiple cell types and signaling pathways. I think the reader will gain a lot from being provided a fuller perspective on past/ contemporary models, not least to appreciate what precisely what progress is represented and/or enabled by the discoveries described here.*

The mechanism to which the Reviewer refers is indeed intriguing and may play a role in both neural and cellular memory. The suggested study is now referenced in the manuscript. However, we would like to point out that because our reporter construct is introduced artificially, its stabilization by ERK at the protein level, while possible, is unlikely to illuminate endogenous mechanisms of memory formation. In our future studies, we aim to monitor not only the reporter transcript, but also transcripts of endogenous CREB-dependent targets, since they may be differentially affected by massed/spaced training.

> *3. The authors find the CRE-Luc-PEST activity remain high 20 hours after spaced stimulation. Is there any evidence that IEGs are transcribed for so long after training in neurons? My understanding is that while LTM and related plasticity can be seen after such long time scales, the direct targets of CREB a(IEGs) are transcribed for much briefer, early time intervals. This should be carefully specified. I stress that it remains very interesting that the fundamental massed/spaced stimulation effect on gene expression is so beautifully reproduced in these cells. But it is important to acknowledge potential differences*

across cells types (which may even exist across neuronal types). I suggest that this be done in a discussion paragraph where the value of this work for future discovery is highlighted.

We would like to thank the Reviewer for raising this excellent point. We agree that this is a difference from conventional neural systems, and that this difference is worth studying in itself. A paragraph has been added to the results acknowledging the difference and providing potential explanations.

4. Please include a short discussion paragraph where the value of this work for future discovery is highlighted.

A paragraph has been added to Discussion.

5. I expect that the older work indicating a role for degradation of the PKA regulatory subunit and sustained PKA activation is "upstream" of the processes being investigated here). Still maybe worth a mention.

Degradation of the PKA regulatory subunit has now been acknowledged as one of the potential mechanisms for the persistence of CREB-dependent transcriptional responses in the Discussion.

6. Page 3 – minor typo "cellular kinase may be involved," (the "be is missing)

The phrase in question has been removed in the revised manuscript.

7. Page 4 (or later): a brief explanation for why only the first and last of the spaced stimulations matter (and not the intermediate ones) in the Aplysia prep will be useful. Particularly since this does not seem to be the case for SHSY5Y cells.

It is not completely accurate to state that "intermediate stimulations don't matter" in *Aplysia*. Rather, in *Aplysia* , a two-trial paradigm with an ITI of 45 and a four-trial paradigm with an ITI of 15 produce identical behavioral results. It doesn't mean that they achieve those results through the same molecular pathways, or that the ITIs or required repetitions must match across systems and agonists. The SH-SY5Y system can be calibrated in such a way so as to induce maximum CRE-luc induction with two pulses only. The experiment here was performed to verify that the observed effect was really a result of a four-trial paradigm, which was successfully confirmed. This was now clarified in the manuscript, and the negative results from two-trial protocols moved to Supplementary.

8. The introduction could be a bit less general and go deeper on a few more sophisticated points (suggested in these comments).

The introduction has been revised, shortened and supplemented with additional references.

9. Page 7: may be clearer to add "for each" so a sentence in the last para would read" "elicited minimal effect after a single pulse of each"

The sentence has been revised accordingly.

10. Page 8 last para similarly perhaps change to:" may not have be possible to observe a potential gene expression correlate of "cellular forgetting".

In our view, the suggested revision implies that our observations do not constitute "true" forgetting, but only its correlate, whereas literal forgetting constitutes something else. In our past theoretical perspectives (Kukushkin and Carew, Neuron 2017; Kukushkin, Neurobiol. Learn. Mem. 2018), referred to in this manuscript, we have argued for a broader, more abstract definition of the terms such as "learning", "forgetting", and "memory" as state-time functions in any homeostatic system. We therefore respectfully believe that the term "cellular forgetting" is sufficiently qualified, and would like to keep it as it stands.

11. Somewhere please clarify whether Forskolin and TPA can substitute for 5HT in Aplysia. (e.g page 9 end of first para)

The manuscript has been modified accordingly.

12. Nuclear translocation of P-ERK is clearly critical for CREB activation. A little more attention to the set up and discussion of these experiments and data will be useful.

The manuscript has been modified accordingly.

Signed: Mani Ramaswami

We would like to thank Prof. Ramaswami for a favorable review and helpful suggestions.

Reviewer #2 (Remarks to the Author):

The authors present an alternative model system to study the molecular mechanisms of the massed-spaced effect of memory formation utilizing the immortalized neuroblastoma cell line SH-SY5Y in conjunction with a short lived CRE-luciferase reporter. First, the authors show that a spaced training paradigm, simulated by stimulation with the PKA and PKC activators forskolin and TPA, is conserved in SH-SY5Y cells. Then the authors show that a spaced training also leads to an increased activation of Erk1/2 kinase compared to control or massed training. Together, these findings suggest that the impact of spaced as opposed to mass training occurs at least in part at the level of intracellular signaling pathways.

Thank you for this assessment.

Major comments

1. It would be helpful for the reader to show a more detailed pathway in Figure 1 depicting the known pathways of how forskolin or TPA lead to ERK and CREB1 activation. Similarly, the authors could provide more information about the known pathways underlying the massed-spaced effect in the introduction in neurons. I would also note that what is missing from Figure 1 is the fact that non-neuronal cells are not islands, but receive signals from other cells, from extracellular matrix, from blood, lymphatics etc. As such, the dichotomy presented feels somewhat simplistic.

Thank you for this suggestion. We agree that a more detailed diagram of cell signaling pathways connecting forskolin/TPA with CREB would be helpful. Because of the complexity of these pathways, we have created a new supplementary figure (Fig. S1) summarizing their key components.

We also agree that Fig. 1 is a simplistic diagram. The intention of this figure is not to provide a detailed overview of the specific signaling network involved in our experiments, but to illustrate a broad relationship between the timescales of computation and their conservation across cell types — a relationship that is certainly complicated by many additional physiological variables not shown in the figure. We addressed this point by acknowledging these additional variables in Fig. S1.

2. To further investigate the molecular mechanism of sustained CRE-luc expression, the authors should reduce Erk1/2 function for example by using an inhibitor or knocking down Erk1/2.

Thank you for this suggestion. We have now performed experiments using U0126, the inhibitor of ERK phosphorylation, which was maintained in culture media for various times during and after training. These experiments confirmed the involvement of ERK in the sustained expression of CRE-luc, and specifically in the spacing effect. Please see Fig. 4A and Fig. S8–S11 in the revised manuscript.

3. The labeling of the Western Blot in Figure 5A is confusing. Does it show t=0 for the three different training paradigms? It appears that a label is missing to indicate the specific training paradigm.

We apologize for the omission of labels in the original version of the manuscript, and thank the reviewer for pointing out the error. The Western blot images have now been replaced with different images from newer experiments that also measured P-CREB alongside P-ERK (Fig. 4A), and labels have been corrected.

4. The authors should explore how increased activation of Erk1/2 leads to sustained CRE-luc expression. Is phosphorylation of CREB1 sustained as well and is this required for the observed effect?

Thank you for this suggestion. We have now performed a series of experiments addressing the role of CREB1 in the spacing effect. Please see Fig. 3–4 and the Results section for a detailed account. Briefly, we showed that CREB1 is

disproportionately phosphorylated following spaced training both immediately after TPA treatments, and at the 24 h time point. We also showed that not only phosphorylation, but total levels of CREB are controlled differentially depending on the training pattern. Finally, we showed that blocking CREB transcriptional output using the inhibitor 666-15 completely blocked the spacing effect.

> *How would increased Erk1/2 activity lead to sustained CREB1 phosphorylation? What other kinases/phosphatases are involved?*

ERK is known to affect CREB phosphorylation through one of the three main pathways: RSK, MSK, and direct phosphorylation. This has now been explained in the legend to Fig. S1. The involvement of other kinases and phosphatases at each stage of the ERK pathway is an excellent question that we are currently investigating. We are especially interested in the control of dual phosphorylation of ERK by differentially distributed phosphatases targeting either one of the activating phosphates in the ERK's TEY motif. However, we believe that the full mapping of this pathway is beyond the scope of this communication, whose present goal is to establish a novel model system.

> *5. To further generalize their observations, the authors should test if they find sustained CRE-luc expression in cells completely unrelated to neurons, for example in HeLa or HEK293 cells.*
>
> *SH-SY5Y are neuroblastoma cells and serum deprivation is the first part of their differentiation protocol. It would be important to determine if other, completely non-neuronal cells would exhibit the same behavior. Without this type of data, I think it's not quite accurate to state that "canonical features of memory… can be embedded in the dynamics of signaling cascades conserved across different cell types," especially since they have only examined a neuroblastoma cell line. Along these lines, isn't it also known from earlier work, including from the Carew lab (Kukushkin et al PNAS 2022), that spaced applications of 5HT alter signaling in isolated sensory neurons (ie that are not part of a neural circuit)?*

The reviewer is absolutely correct in pointing out that cultured neurons are known to show differential responses to spaced and massed applications of agonists (5HT or KCl). The introduction now explicitly acknowledges these studies as foundational to our work. Our present study is the next step along the same path of abstracting cell-autonomous features of memory and demonstrating that even non-neural cells possess similar pattern-detecting properties. The reviewer also raises a valid point that neuroblastoma cells are not completely abstracted from the nervous system, and in fact could be in the process of acquiring neuron-like qualities due to serum deprivation. For this reason, we have now validated our key findings in HEK293 cells. As in SH-SY5Y cells, both ERK and CREB were shown to be disproportionately phosphorylated in HEK293 cells immediately after spaced training. HEK293 cells stably expressing CRE-luc also showed disproportionate induction of luciferase 24 h after spaced training compared to massed training. See Fig. 4C–D in the revised manuscript.

6. The discussion is very short and should be elaborated on. What are the potential pathways that lead to persistent CRE-luc expression? What is known about these pathways in neuronal cultures and tissues?

The discussion has now been expanded and includes the overview of potential mechanisms of establishing persistence of CRE-luc expression. We have also provided a detailed description of potential pathways in play in Fig. S1. Our future studies, based on the model system reported here, will elaborate further on specific elements in this signaling network.

Minor comments
1. The authors did not define most abbreviations used in the manuscript. Please correct.

Thank you for pointing this out. Abbreviations have been defined throughout.

2. In the paragraph describing Figure 3 the sentence below should be as followed:

The most dramatic differences between the effects of a single pulse and repeated pulses (29% vs 381% compared to untreated controls after 24 h, respectively) were observed for TPA, which is especially striking since the length of TPA treatment had hardly any effect of luciferase expression (Fig. 2).

The most dramatic differences between the effects of a single pulse and repeated pulses (29% vs 391% increase compared to untreated controls after 24 h, respectively) were observed for TPA, which is especially striking since the length of TPA treatment had hardly any effect of luciferase expression (Fig. 2).

The manuscript has been modified accordingly.

3. It was not clear what vehicle Foskolin and TPA were dissolved in prior to adding to the media, and/or whether this solvent was also used as a control (eg if it was DMSO or ethanol).

Thank you for pointing out this omission. All drugs used for cell treatments were dissolved in DMSO and diluted in media to a final DMSO concentration of 0.1%. This has been made clear in the Methods section.

Reviewer #3 (Remarks to the Author):
In this manuscript, the authors mimicked training using repeated pulses of forskolin and TPA, and measured CRE-luciferase reporter gene expression at various points. They developed a short-lived reporter system from the human neuroblastoma cell line (SH-SY), stably transfected with a CRE-dependent, PEST-modified luciferase gene. Four spaced pulses of either agonist elicited stronger and more sustained luciferase expression than a massed treatment, demonstrating a form of temporal pattern discrimination. Spaced pulses also

resulted in stronger phosphorylation and nuclear translocation of ERK, indicating that the temporal pattern, at least in part, is decoded either by ERK itself or its upstream kinases, suggesting that canonical features of memory do not necessarily depend on neural circuitry, but can be embedded in the dynamics of signaling cascades conserved across non-neuronal cell types. The experimental design and data interpretation are sound and the manuscript was well written. I recommend its publication after improving the following minor points.

Thank you for this assessment.

1. Citation: A similar massed vs. spaced reporter gene assay was done previously using Aplysia neurons which employed serotonin (5HT) as the memory-inducing stimulus. It could be referred in introduction or discussion. -Kaang BK, Kandel ER, Grant SG. Neuron 10:427 (1993)

Thank you for this suggestion. The citation has been added in the Introduction.

2. The authors argue that their reporter cell line can substitute a neuronal system in some aspects and can be used for the development of predictive models of memory formation. However, their message needs to be tone down. They may also discuss the limitations as the non-neuronal cell system apparently lacks synapses, synaptic plasticity and a cross talk between cell soma and synapse, etc.

The manuscript has been revised extensively and the tone has been moderated throughout.

3. Typo in line 44: may involved in..

The sentence in question has been removed.

In conclusion, we again thank the reviewers for their thoughtful and constructive comments and suggestions. We feel that the manuscript has been significantly improved by virtue of our response to these comments, as well as by the additional experiments suggested by the reviewers.

Best regards,

Nikolay V. Kukushkin

Thomas J. Carew

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript has been comprehensively and carefully revised. I congratulate the authors on a very nice and interesting study!

We would like to thank Reviewer 1 for the favorable assessment and constructive criticism.

Reviewer #2 (Remarks to the Author):

This revised application is improved with the inclusion of experiments done in HEK293 cells, inclusion of experiments with MEK inhibitors and experiments examining CREB phosphorylation. It represents a reductionist approach to understanding the impact of spaced versus massed stimulation on memory formation, which, as the authors point out, can be used to guide "formalized molecular models of memory formation."

Thank you for this favorable assessment.

A couple of comments:

In figure 1, it's not clear if the timing on the y axis of the panel on the left refers to timing of functional impact of the process or to the timing of the process itself. Changes in gene expression, for example, can occur over a time scale of minutes. Regarding my earlier comment about the simplicity of figure 1, I was not referring to the type of simplification of signaling pathways that is now provided in supplemental figure 1 (which I do not think is necessary), but rather to the fact that the synaptic signaling that is considered "neuron-specific" in the right panel has clear parallels in non-neuronal signaling in the form of temporal patterns of extracellular signaling.

Both the left and the right panel generalize over a vast number of cellular processes and as such, refer to the order-of-magnitude timescales over which deviations in homeostasis induced by various types of factors typically occur. The Reviewer is correct in pointing out that cellular processes are not categorically separated by their timing, and neither are neuronal and non-neuronal processes. However, our diagram clearly reflects these considerations by representing both dimensions as *gradients*. We have provided the theoretical basis for such generalization in Kukushkin and Carew, Neuron 2017, a foundational paper in our lab that is referred to in the introduction of the manuscript. The point illustrated by the figure is *relative*: neurons generally have to deal with signaling events on much shorter timescales than non-neural cells; however, as our study shows, even non-neural cells can discriminate patterns on the time scales of minutes, highlighting precisely the parallels between neural and non-neural cells that the Reviewer points to. It is a necessary point to make because non-neural cells are

rarely (if ever) subjected to stimulus patterns at such short time scales, underscoring the novelty of our approach.

> *It's important to note that both TPA and elevations of cAMP, including by forskolin, differentiate SH-SY5Y cells into neurons (Kovalevich and Langford 2013, Monaghan et al 2008), and as such the vehicle treated control is not quite right as the TPA and forskolin treated cells are undergoing differentiation while the vehicle treated are not.*

The reviewer had already raised the point about potential differentiation by serum deprivation in the previous review, requesting validation of our data in a different, non-neuroblastoma cell line. We have noted this concern in the revised manuscript, and for this reason, created a new reporter cell line based on embryonic kidney cells, HEK293, completely validating all our key results in this cell line, which behaved in the exact same way as SH-SY5Y. Even if differentiation into neurons in SH-SY5Y cells overlapped in its molecular mechanics with transcriptional memory that we observe (which it may very well do — many mechanisms of memory are a re-engagement of mechanisms of development), it would not in any way compromise the validity or interpretation of our data, since, as we now directly show, the exact same effects are clearly observed in kidney cells. Since the concern has already been noted and eliminated by direct control experiments, we do not see a valid reason to amend our manuscript, or conditionalize our results as “not quite right”.

> *Reviewer #3 (Remarks to the Author):*
>
> *The authors have addressed my concerns adequately.*

We would like to thank Reviewer 3 for the favorable assessment and constructive criticism.

In conclusion, we again thank the reviewers for their thoughtful and constructive comments and suggestions. We believe that we have already addressed remaining comments by Reviewer 2, and so respectfully ask that the manuscript be published without modification from the previous version.

Best regards,

Nikolay V. Kukushkin

Thomas J. Carew