Comment on, **"Multiple repressive mechanisms in the hippocampus during memory formation"**

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Abstract.

Cho *et al.* (Reports, 02 October, p.82) report that gene repression following contextual fear conditioning regulates hippocampal memory formation. We observe low levels of expression for many of the top candidate genes in the hippocampus, and robust expression in choroid plexus, as well as repression at 4 hours following contextual fear conditioning, suggesting the inclusion of choroid plexus mRNAs in Cho *et al.* hippocampal samples.

Main text.

The molecular mechanisms regulating the acquisition and storage of memory continue to be the focus of intense study. Recently, Cho *et al.* (*1*) investigated gene transcription (by RNA-Seq) and translation (by ribosome profiling) in the hippocampus in response to contextual fear conditioning in mice. The authors report repression of multiple transcripts and ribosome-associated mRNAs in the hippocampus up to 4h following foot shock as compared to naïve controls. They propose that experience-mediated regulation of transcription and translation of this set of genes, through suppression of estrogen receptor signaling, may be central for the molecular mechanisms of hippocampal memory. We re-examined the top 15 differentially-expressed genes (DEGs) reported by Cho *et al.* (*1*) at 4h following contextual fear conditioning, and observed very low levels of expression in the hippocampus [see Allen Brain Atlas (*2*)], but noted that 11 of the genes rank among the top expressed transcripts in the choroid plexus (ChP), and together belong to the characteristic transcriptomic signature of the ChP that we (*3*), and others (*4*) have characterized.

The ChP is an epithelial cell layer that extends into the brain's ventricles and is the principal source of cerebrospinal fluid (CSF) (*5*). In the lateral ventricles, the ChP develops from the medial cortical wall, ventral to the hippocampus. In the mature brain, the ChP develops papillary structures that fill the ventricular space, lying in close proximity to the hippocampus (**Fig. 1A**). The ChP appears translucent and unless explicit care is taken when dissecting the hippocampus, inclusion of the ChP as part of the hippocampal preparation is inevitable, thereby potentially confounding the analysis of the transcriptome.

In light of the substantial consequences that inadvertent ChP inclusion in the hippocampal samples would have on the interpretation of the data, we attempted to replicate the findings of Cho *et al.* (*1*). Adult C57BL/6N male mice (the strain used by Cho *et al.* [*1*]) were

subjected to foot shock to contextually condition a fear response, or left untreated. After 4h, the hippocampus as well as the ChP in the lateral and fourth ventricles were concurrently isolated from trained and control mice. We performed quantitative PCR (qPCR) of 10 transcripts from the list of the top 15 DEGs at 4h post foot shock [see Cho *et al.* (*1*) Table S2]. The expression of these 10 genes was 100 to 20,000 fold higher in the ChP as compared to the hippocampus (**Fig. 1B**). As controls, we also measured *Gapdh* normalized mRNA levels of 3 hippocampus-specific genes [hippocampal expression: *Neurod6* (8.2%)*, Frzb* (1.6%)*, and Trpc6* (0.5%) *(6)*] not highly expressed in the ChP [ChP expression: *Neurod6* (0.005%)*, Frzb* (0.1%)*, and Trpc6* (0.001%) *(6)*].

To compare the expression levels of the 10 ChP signature genes in our hippocampal samples to the Cho *et al.* (*1*) hippocampal samples, we normalized the data to *Neurod6* (*6*). The expression of these genes in our samples showed 0.02-1.4% enrichment, demonstrating their low expression in the hippocampus (**Fig. 1C**). In contrast, the hippocampal RNA expression values deposited by Cho *et al.* (*1*) in the Gene Expression Omnibus [GSE72064], suggested enrichment of these ChP signature transcripts (7-36% of *Neurod6* mRNA levels; **Fig. 1C**). Together, these results point to the presence of ChP-derived mRNA in the Cho *et al.* (*1*) hippocampal samples at 4h following contextual fear conditioning.

Given these gene enrichment patterns, we were compelled to evaluate the exciting but unexplored possibility that contextual fear conditioning causes rapid changes in gene expression in the ChP, possibly to a degree exceeding changes in the adjacent hippocampus. To test this prediction, we measured the mRNA levels of the 10 genes in lateral and fourth ventricle ChP and found that their expression levels were suppressed in both tissues following foot shock as compared to time-matched controls, with reduced expression of *Sostdc1*, *Augurin (1500015O10Rik, Ecrg4)*, and *Kcne2* reaching statistical significance (**Fig. 1D, E**). In agreement with Cho *et al.* (*1*), the expression levels of several genes (*Aqp1, Kcne2, Tmem72, Slca5*) were reduced in our hippocampal samples following foot shock (**Fig. 1F**). However, the overall expression levels of these genes were low with respect to *Gapdh* (**Fig. 1F**). Hippocampal expression of *Sostdc1* did not change, while the expression of *Cldn2* increased in response to foot shock (**Fig. 1F**). Taken together, our data suggest that the unintended presence of ChP mRNAs in the Cho *et al.* (*1*) hippocampal samples resulted in robust gene expression changes at 4h following contextual fear conditioning, which cannot be attributed solely to the hippocampus.

Indeed, our results suggest that the most robust responses are attributable to the ChP itself. We did not directly compare ChP and hippocampal gene expression at other times post-conditioning; therefore it is unknown whether inclusion of ChP occurred for other time points.

While it remains to be determined if transcriptional repression of these genes in the ChP is functionally important for memory formation, it is clear that the ChP transcriptome rapidly responds to stressful stimuli including contextual fear conditioning. Indeed, foot shock activates the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, with sudden increases in blood pressure, heart rate, and body temperature (*7, 8*), all stimuli known to affect the ChP at the blood-CSF barrier.

Recently Stankiewicz *et al.* (*9*) reported acute stress-induced reduction in expression of a set of genes in hippocampal samples that overlap with those reported here (**Fig. 1**) and in the Cho *et al.* (*1*) DEG set. The authors reported that these genes represent the ChP transcriptome and cautioned against ChP inclusion in hippocampal samples (*9*). Sárvári *et al.* (*10*) observed that administration of estradiol to ovariectomized female rats increased levels of multiple ChP-signature transcripts in hippocampal samples. Consistent with Sárvári *et al.* (*10*), Cho *et al.* (*1*) observed reduced expression of most of these genes, including *Ttr* (Cho *et al.* Fig. 2D), following peripheral administration of an estrogen receptor antagonist. While we did not repeat these pharmacological studies, prior investigations have demonstrated direct actions of estrogens on ChP transcription of *Ttr* (*11*), its most highly expressed gene (*3,4*).

In summary, the probable inclusion of ChP mRNAs in hippocampal samples in Cho *et al.* (*1*; and likely in studies from other groups) provides an alternative location for the source of the genes showing the greatest changes in expression following contextual fear conditioning. Our study re-examined gene expression at 4h following fear conditioning; it is unknown whether the rapid translational and transcriptional changes observed at the other time points examined by Cho *et al.* (*1*) also reflect contributions from the ChP. An interaction between the ChP and hippocampal cognitive function has been suggested (*12*). Changes in protein secretion by the ChP, due either to translational or transcriptional changes, could contribute to the mechanisms of hippocampal memory formation. Our study provides a reference guide for the research community with which enrichment of ChP signature genes in hippocampal samples can be used to evaluate the purity of the isolated starting material. Furthermore, our results reveal that the ChP rapidly responds to the environment by altering mRNA levels of some of its most highly

expressed genes. These transcriptomic events may ultimately contribute to changes in CSF composition, thereby influencing brain function.

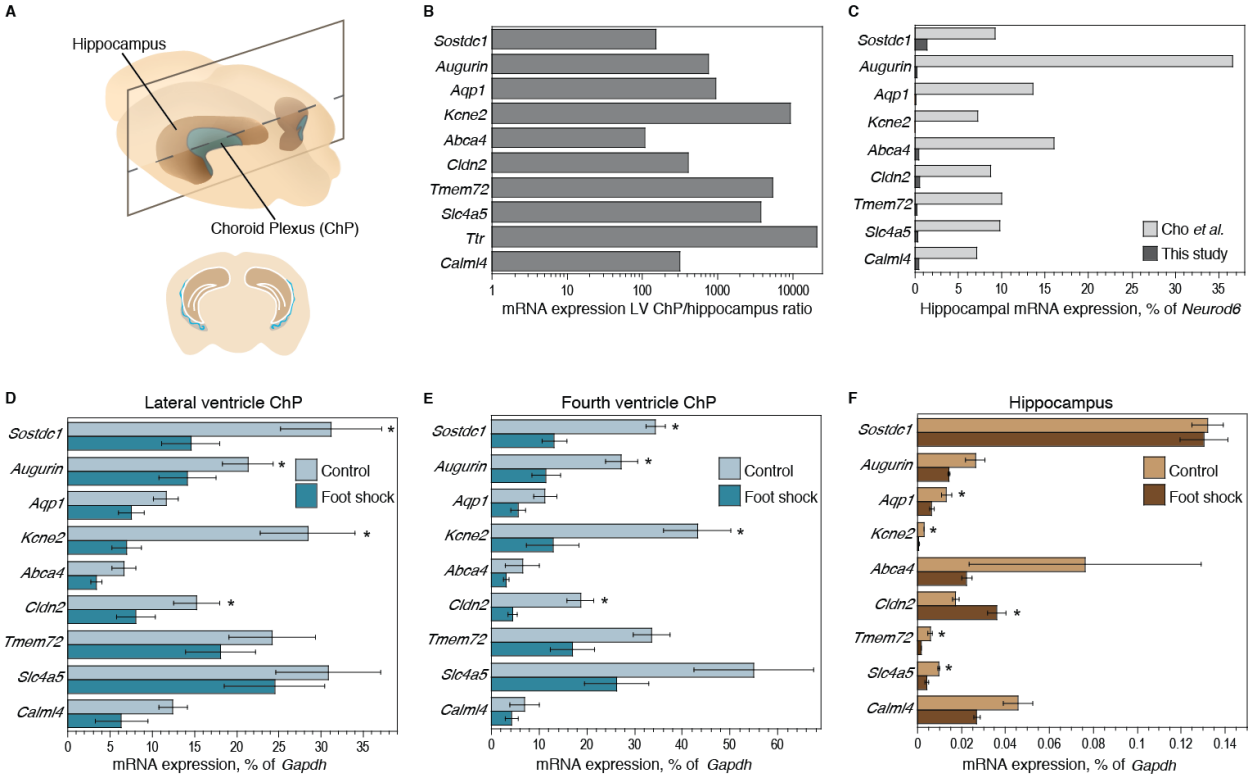

Figure 1. Gene expression analyses in choroid plexus and hippocampus reveal tissue-specific responses to contextual fear conditioning. (**A**) The ChP of the lateral ventricle (blue) is proximate to the hippocampus (brown), requiring specialized dissection procedures to isolate and separate the tissues. (**B-F**) C57BL/6N 9-week old male mice, treated in accordance with the protocol approved by the IACUC of Boston Children's Hospital, were individually housed in a 12h light/dark cycle (7:00/19:00) and handled daily for 3 minutes each, for four consecutive days. Following handling on day 4 (at 10:00), mice were either returned to their home cage (naïve mice), or exposed to foot shock (148 seconds in chamber, 0.6 mA shock for 2 seconds, 30

seconds in chamber, returned to home cage at 180 seconds). Four hours later (at 14:00), ChP and hippocampus from pairs of naïve and trained mice were concurrently isolated, ensuring that each trained mouse had an exact time-matched naïve control. Tissue RNA was purified, analyzed by qPCR (TaqMan, Applied Biosystems, n=3, with each gene-specific measurement and standard performed in triplicate), and the data were used to generate panels B-F. (**B**) The expression levels of 10 selected mRNAs out of the top 15 differentially-expressed genes reported by Cho *et al.* (*1*) are 100-20,000 fold higher in the ChP than in the hippocampus (n=12). (**C**) High expression of ChP-specific transcripts in the hippocampal samples used by Cho *et al.* (*1*). The expression levels of the selected genes in the hippocampal samples were normalized to *Neurod6* using material generated in this study (n=12) and by analysis of the Cho *et al.* (*1*) data in the GEO Dataset GSE72064 (n=15) [*Ttr* levels are not plotted; the values are 12% in this study and 3170% in Cho *et al.* (*1*)]. (**D-F**) mRNA levels 4h after foot shock in the lateral and fourth ventricle ChP and in the hippocampus. Data are represented as mean ± S.E.M.; n=3; *$p<0.05$, one-way ANOVA with repeated measures for ChP samples, t-test for hippocampal samples. *Ttr* expression was not affected by foot shock in any of the tissues. Of note, since both the ChP (*13*) and hippocampus (*14*) demonstrate circadian gene expression patterns, the magnitude of gene expression changes could vary as a function of time of day used for the naïve control, as well as the time of day that the experiment was performed.

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References and Notes

1. J. Cho *et al., Science* **350**, 82 (2015).
2. http://mouse.brain-map.org
3. M. P. Lun *et al.*, *J Neurosci* **35**, 4903 (2015).
4. S. A. Liddelow *et al.*, *PLoS One* **8**, e65629 (2013).
5. M. P. Lun, E. S. Monouki, M. K. Lehtinen, *Nat Rev Neurosci* **16**, 445 (2015).
6. E. S. Lein, X. Zhao, F. H. Gage, *J Neurosci* **24**, 3879 (2004).
7. B. S. McEwen *et al., Nature Neurosci* **18**, 1353 (2015).
8. R. S. Thompson, P. V. Strong, and M. Fleshner, *Behav Sci* **2**, 57 (2012).
9. A. M. Stankiewicz, J. Goscik, A. Majewska, A. H. Swiergiel, G. R. Juszczak, *PLoS One* **10**, e0142195 (2015).
10. M. Sarvari *et al.*, *Endocrinology* **156**, 2632 (2015).
11. T. Quintela *et al*., *Cell Mol Neurobiol* **29**, 475 (2009).
12. K. Baruch *et al., Science* **346**, 89 (2014).
13. T. Quintela, C. Sousa, F. M. Patriarca, I. Goncalves, C. R. A. Santos, *Brain Struct Funct* **220**, 1251 (2015).
14. K. L. Eckel-Mahan *et al., Nature Neurosci* **11**, 1074 (2008).