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Analysis of hippocampal transcriptomic responses to technical and biological perturbations

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Abstract Cost-effective next-generation sequencing has made unbiased gene expression analysis possible. Single-neuron gene expression studies may be especially important for understanding nervous system structure and function because of the neuron-specific functionality and plasticity that defines functional neural circuits. Cellular dissociation is a prerequisite technical manipulation for single-cell and single cell-population studies, but the extent to which the cellular dissociation process cells affects neural gene expression has not been determined, nor has it been determined how gene expression is altered by the stress that accompanies many of the behavioral manipulations that are required to study learning and memory and other cognitive functions. Here, we determined to which extent cellular dissociation-induced changes in hippocampal gene expression might confound studies on the behavioral and physiological functions of the hippocampus. We processed tissue punch samples from the dentate gyrus (DG), CA3, and CA1 hippocampus subfields using either a tissue homogenization protocol or a cellular dissociation protocol in preparation for RNA sequencing analysis to evaluate the impact of the tissue preparation. Then, we evaluated the effect of stressful experience and cognitive training on hippocampus subfield specific gene expression and determined to which extent these response overlap with the cellular dissociation response. Finally, we assessed the extent to which the subfield-specific gene expression patterns are consistent with those identified in a recently published hippocampus subfield-specific gene expression database. We report substantial differences in baseline subfield-specific gene expression, that 1% of the hippocampal transcriptome is altered by the process of cellular dissociation, that an even weaker alteration is detected 24 h after stressful experience, and that while these alterations are largely distinct from the subfield specific response of the hippocampus transcriptome to cognitive training, there is nonetheless some important confounding overlap. These findings of the concordant and discordant effects of technical and behavioral manipulations should inform the design of future neural transcriptome studies and thus facilitate a more

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comprehensive understanding of hippocampal function.

Introduction

Nervous systems are comprised of diverse cell types that include neurons, glia, and vascular cells, each serving distinct functions and thus expressing different genes. Consider the hippocampus, a structure central for spatial navigation and the processing of event memory in the mammalian brain. To date, distinct aspects of navigation and memory processing have been firmly correlated to activity of particular cellular subfields within the hippocampal formation. This subfield-specific understanding of hippocampal function, has led to the notion that cells within a given subfield are homogeneous in their molecular blueprint and perform the same function. However, even within the anatomically-defined subfield of CA1, there are identifiable subclasses of pyramidal cells that belong to distinct functional circuits (***Mizuseki et al., 2011***; ***Danielson et al., 2016***). This diversity is even greater when we consider that specific cells within a functional class can be selectively altered by neural activity in the recent or distant past. For example, only a third of the pyramidal cells of the superficial CA1 sub-layer are expected to be meaningfully active during experience of a particular environment and only a subset of those might have been sufficiently engaged to alter the strength of a synapse which then triggers further gene expression changes within the functional class(***Guzowski et al., 1999***, ***2006***). All this diversity implies distinctive gene expression, very likely at the level of single neurons, and such considerations may strongly curtail interpretations of gene expression studies that use mixtures of cells or microdissected tissue samples.

Fortunately, recent advances in tissue harvesting and processing, as well as in sequencing technologies have allowed detailed analyses of genome-scale gene expression profiles at the level of single cell populations, in the context of brain and behavior studies (***Chalancon et al., 2012***; ***Harris and Hofmann, 2014***; ***Mo et al., 2015***). These approaches have led to systems-level insights into the molecular substrates of neural function, along with the discovery or validation of candidate pathways regulating physiology and behavior (***Cembrowski et al., 2016a***). While the complexity of some tissues complicates the interpretation of transcriptome data collected from samples containing hundreds to tens of thousands of cells representing numerous cellular subclasses at different levels of diversity, difficulties with interpretation can be minimized by careful experimental design governing both data collection and data analysis. To complement this effort, and optimize experimental designs, it is necessary to understand the extent to which the treatment of tissue samples prior to transcriptome analysis might confound interpretation of the results.

We examined the effect of cellular dissociation on the transcriptomes of specific hippocampal subfields (CA1, CA3, and DG) by comparing tissue homogenization (as a control) and cellular dissociation protocols. We then examined and compared the effect of prior stressful experience that accompanies many protocols to assess learning, memory and innate behaviors, and cognitive training on hippocampal subfield gene expression. Finally, we compared these results to a public data set of cell type-specific hippocampus gene expression to further validate the patterns of gene expression that we identified. Knowing how technical perturbations influence the ability to detect the molecular signature of differences in neural and behavioral variables is an important step in calibrating the ability to mechanistically understand hippocampal function. In addition to understanding the impact of cell dissociation and stressful experience on hippocampus gene expression, the present findings allow evaluating the extent to which gene expression profiles of heterogeneous tissue samples compare with single neuron population gene expression profiles.

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Methods and Materials

Animals

All animal care and use complies with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the New York University Animal Welfare Committee and the Marine Biological Laboratory Institutional Animal Care and Use Committee. C57BL/6J mice were used, a generous gift from the Jackson Laboratory (Bar Harbor, ME). All mice were housed on a 12:12 (light:dark) cycle with continuous access to food and water.

Tissue preparation

Each mouse was taken from its cage, anesthetized with 2% (vol/vol) isoflurane for 2 minutes and decapitated. Transverse 300 µm brain slices were cut using a vibratome (model VT1000 S, Leica Biosystems, Buffalo Grove, IL) and incubated at 36°C for 30 min and then at room temperature for 90 min in oxygenated artificial cerebrospinal fluid (aCSF in mM: 125 NaCl, 2.5 KCl, 1 $MgSO^4$, 2 $CaCl^2$, 25 $NaHCO^3$, 1.25 NaH^2PO^4 and 25 Glucose) (Pavlowsky and Alarcon, 2012; Pavlowsky et al., 2017). Tissue samples were collected from CA1, CA3, and DG, respectively in the dorsal hippocampus by punch (0.25 mm, P/N: 57391; Electron Microscopy Sciences, Hatfield, PA). All punches for RNA sequencing came from the slice corresponding to image 74 of the Allen Brain Reference Atlas (RRID:SCR_013286).

Animal and tissue preparation for assessing impact of cellular dissociation

A 1-year-old female C57BL/6J mouse was used for the cellular dissociation experiment. One tissue punch was designated for the control homogenized processing and the other for the cellular dissociation treatment (Fig 1A). Two adjacent tissue samples were collected from each subfield for each mouse. The 'control sample' was processed using the manufacture instructors for the Maxwell 16 LEV RNA Isolation Kit (Promega, Madison, WI). The 'cellular dissocation sample' was incubated for 75 minutes in aCSF containing 1 mg/ml pronase at room temperature, then vortexed and centrifuged. The incubation was terminated by replacing aCSF containing pronase with aCSF. The sample was then vortexed, centrifuged, and gently triturated by 200-µl pipette tip twenty times in aCSF containing 1% FBS. The sample was centrifuged and used as input RNA isolation using the Maxwell 16 LEV RNA Isolation Kit (Promega, Madison, WI).

Animals and tissue preparation for assessing impact of stressful experience and cognitive training

Male C57BL/6J mice that were 3-4–months old were used. They were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed at the Marine Biological Laboratory. Gene expression in tissue from mice taken from the home cage was compared to mice that received mild foot shock, to evaluate how gene expression is affected by stressful experience, which is a common confound of behavioral manipulations such as water maze learning, fear conditioning, inhibitory avoidance, active place avoidance and other learning and memory test paradigms. The mice were placed on an elevated circular 40-cm diameter arena that rotated at 1 rpm. The arena wall was transparent and so contained the mice to the arena and allowed it to observe the environment.

Mice in the stressful experience group received a short series of unavoidable mild foot shocks while walking on the arena. Each shock was a constant current 0.2 mA 500 ms 60 Hz shock. The time series of shocks matched the shock time series from training in an active place avoidance task. Each session was 10 minutes and the mice had three sessions a day for 3 days with an inter-trial interval of 2 hours, during which it was returned to the home cage. The shock protocol is initially stressful, assessed by elevated plasma corticosterone, but by the second day, corticosterone levels return to baseline levels (Lesburguères et al. 2016). The mice received an average of 8 shocks per day with a maximum of 29 and a minimum of 1 shock.

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Comparisons were made to home cage control mice that were not exposed to the arena.

Mice in the cognitive training and yoked control groups were placed on the rotating arena and trained in the active place avoidance task. The task conditions the mice to avoid foot shock that can be localized using extramaze visual cues. Acquiring and remembering the avoidance requires intact hippocampus function and long-term synaptic potentiation (Cimadevilla et al., 2001; Hsieh et al., 2017; Pavlowsky et al., 2017). Yoked control mice were exposed to the arena the same amount of time as the cognitive training and they received the identical time series of foot shocks as an animal in the cognitive training group. Thus the yoked mice experienced the same environment as the trained mice but for the yoked controls, shock was unavoidable. While the trained and yoked animals received the same number of shocks, only the trained animals exhibited an avoidance response and only they yoked animals exhibited a detectable stress response (Lesburgueres et al., 2016).

Twenty-four hours after the behavioral manipulations the mice were sacrificed and tissue punches were collected was described above.

RNA sequencing

RNA from CA1, CA3, and DG was isolated using the Maxwell 16 LEV RNA Isolation Kit (Promega, Madison, WI). RNA libraries were prepared by the Genomic Sequencing and Analysis Facility at the University of Texas at Austin using the Illumina HiSeq platform.

Raw reads were processed and analyzed on the Stampede Cluster at the Texas Advanced Computing Facility (TACC). Quality of the data was checked using the program FASTQC. Low quality reads and adapter sequences were removed using the program Cutadapt (Martin, 2011). We used Kallisto for read pseudoalignment to the Gencode MV11 mouse transcriptome and for transcript counting (Mudge and Harrow, 2015; Bray et al., 2016). Transcript counts were converted into gene counts using the reshape2 R package (***Wickham, 2016***).

We downloaded the gene counts from the ***Cembrowski et al.*** (***2016b***) dataset archived (NCBI GEO:GSE74985) (***Cembrowski et al., 2016b***). Briefly, this data set contains hippocampal gene expression data for pools of 112 ± 6 cells for each of 5 cell types (CA1, CA2, and CA3 pyramidal neurons and DG mossy and granule cells) from behaviorally naive, transgenic mice that express a fluorescent protein label in the specific cell types. The DG, CA1, and CA3 cells were manually sorted from both dorsal and ventral slices and fluorescently-labeled neurons were manually collected, DG granule cells from Rbp4-Cre KL100, CA3 pyramidal cells from Mpp3-Cre KG118, and CA1 pyramidal cells from Vipr2-Cre KE2 mice.

Statistical analyses

Gene-level counts were were imported into R for reproducible data management, manipulation and analysis using the dplyr, plyr, and knitr packages (***Xie, 2015***, ***2014***, ***2017***; ***Wickham and Francois, 2016***).

We used DESeq2 for gene expression normalization and quantification of gene level counts (***Love et al., 2014***). We used a threshold of a false discovery corrected (FDR) p value < 0.1. Statistics on the principal component analyses (PCA) were conducted in R (***Wickham, 2009***, ***2011***). We used the VennDiagram R package (***Schwenk et al., 1984***) for preliminary visualization of differential gene expression, but the final Venn diagrams were drawn with Adobe Illustrator.

The hierarchical clustering analysis was conducted and visualized using the R package pheatmap (***Kolde, 2015***) with the RColorBrewer R packages for color modifications (***Neuwirth, 2014***). The bootstrap probability values for the dendrogram were calculated using the R package pvclust (***Suzuki and Shimodaira, 2006***).

PCA was conducted in R using the DESeq2 and genefilter R packages (***Love et al., 2014***; ***Gentleman et al., 2017***). The PCA analysis was visualized using the ggplot2, cowplot, and RColorBrewer R packages (***Wickham, 2009***; ***Wilke, 2016***).

We used GO_MWU for analysis of GO ontology (***Wright et al., 2015***). Figure 2 was generated

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using –log(p-value) as a continuous measure of significance to identify GO categories that are significantly enriched with either up- or down-regulated genes. No significance cutoff is required for the analysis, but an arbitrary p-value was set to visualize the top 10 most significant GO terms. Figure 5 was generated in two steps. First, a p-value = 0.1 was set for determining significantly expressed genes in each analysis, and these data were converted into a binary (0 or 1) for a typical GO enrichment analysis using Fisher's exact test to determine if GO categories are overrepresented among the significantly expressed genes.

Archival of data, code, and figures

The raw sequence data and intermediate data files are archived in NCBI's Gene Expression Omnibus Database (accession numbers GSE99765 and GSE100225). The data and code are available on GitHub https://github.com/raynamharris/DissociationTest, with an archived version at the time of publication available at Zenodo (***Harris et al., 2017a***). The schematic images and figure modifications were made using Adobe Illustrator and archived in FigShare under a CC-BY license (***Harris et al., 2017b***).

Results

We obtained an average of 5 million reads for each hippocampal tissue sample and quantified the expression representing 22,485 genes in the mouse reference transcriptome MV11.

The effects of cellular dissociation on hippocampal transcriptomes

We identified 162 genes that were differentially expressed between the control and dissociated samples, 331 genes that were differentially expressed genes (DEGs) between any of the three hippocampus subfields, and 30 genes were shared between both sets of differentially expressed genes at p-value < 0.05 (Fig. 1A,B).

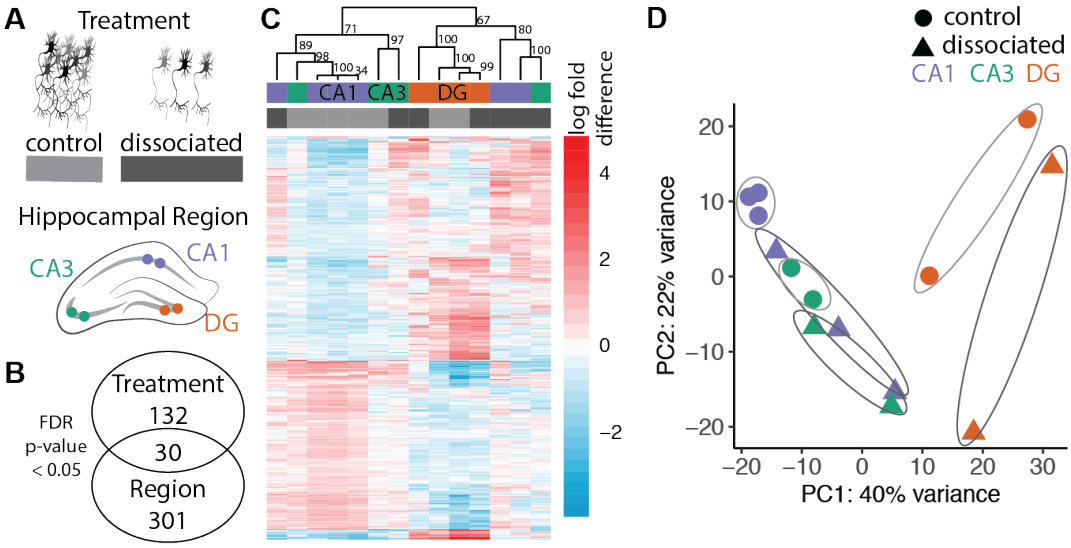

Figure 1. The effect of cellular dissociation on hippocampal transcriptomes. A) From a single female mouse, we collected 2 CA1, CA3, and DG hippocampal tissue samples. One sample was subjected to a cellular dissociation treatment (dissociated) whereas the control samples (control) were standardly homogenized. **B)** We identified 162 dissociation-induced changes in gene expression, 331 genes with region-specific expression patterns, and 30 genes differentially expressed by both region and treatment (FDR p-value < 0.05). **C)** Hierarchical clustering separates the hippocampal subfields of the homogenized samples (light gray) but not the dissociated samples (dark gray). **D)** PC1 accounts for 40% of all gene expression variation and by inspection, separates the DG samples from the CA1 and CA3 samples. PC2 accounts for 22% of the variation in gene expression and varies significantly with treatment. Ellipses are hand-drawn.

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A hierarchical clustering analysis of all differentially expressed genes does not give rise to distinct clusters that are separated by subfield or method; however, when examining the control, homogenized samples alone (identified with light grey boxes), the three subfields form distinct clusters, while the dissociated samples do not cluster by subfield (Fig. 1C).

Next, we conducted a principal component analysis of all identified genes. PC1 accounts for 40% of the variation and visually separates the DG samples from the CA1 and CA3 samples (Fig. 1D). To confirm statistical significance of this visual pattern, we conducted a two-way treatment x region ANOVA and confirmed a significant effect of region ($F_{2,11}$= 17.69; p = 0.0004). Post hoc Tukey tests confirmed CA1 = CA3 < DG. The effect of treatment and the treatment x region interaction were not significant. PC2 accounts for 22% of the variation in gene expression and varies significantly with treatment ($F_{1,12}$=6.125; p = 0.03) but not by region or the treatment x region interaction. None of the other PCs showed significant variation according to either region or treatment.

The effects of stressful experience on hippocampal transcriptomes

We examined the effect of stressful experience, which is a common confound of behavioral manipulations because animals in different experimental groups often experience different levels of stress, especially if the experimental procedure is not intentionally stressful. We identified 0 genes that were significantly expressed between samples from the home cage and shocked mouse samples; 1669 genes were significantly differentially expressed between any of the three brain regions at p-value < 0.05 (Fig. 2A, B).

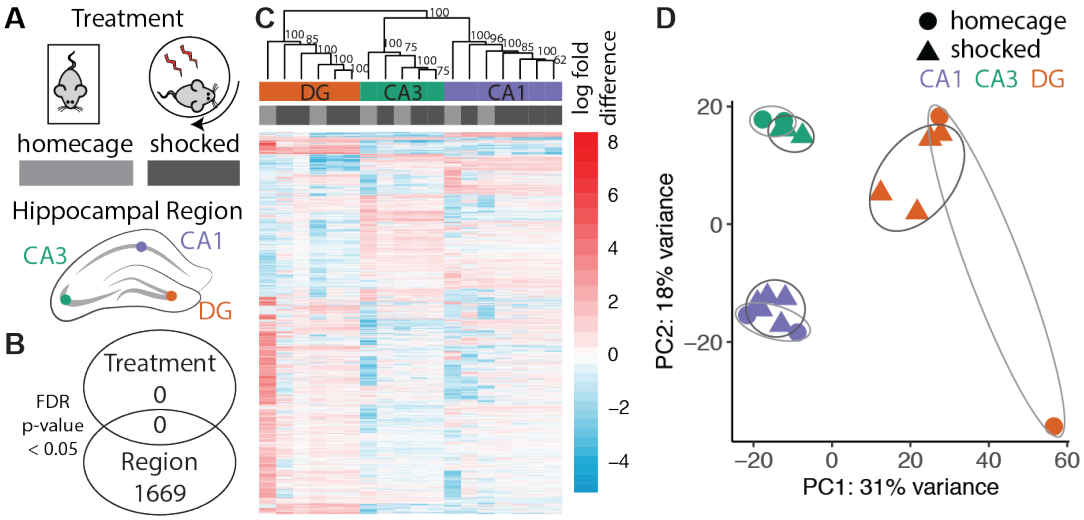

Figure 2. The effects of a stressful experience on hippocampal transcriptomes. A) We compared CA1, CA3, and DG tissue samples from control mice taken directly from their home cage to mice that were subjected to a mild foot shock. **B)** We identified 0 genes that responded to treatment, and 1669 genes that were differentially regulated across regions of the hippocampus (FDR p-value < 0.05). **C)** Hierarchical clusters groups samples by brain region but distinct treatments clusters are not present. **D)** PC1 accounts for 31% of the variation and visually separates the DG samples from the CA1 and CA3 samples. PC2 accounts for 1% of the variation and distinguish the three subfields. Ellipses were hand-drawn.

Hierarchical clustering of the differentially expressed genes gives rise to three distinct clusters corresponding to the three subfields, with CA1 (purple) and CA3 (green) being more similar to one another than to DG (orange), whereas the effects of the stress manipulation were not distinctive (Fig. 2C).

Next, we conducted a principal component analysis of all the genes that were measured. PC1 accounts for 31% of the variation and by inspection, separates the DG samples from the

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CA1 and CA3 samples (effect of region: $F_{2,15}$= 42.89; $p < 0.001$; Fig. 2D). Post hoc Tukey tests confirmed CA1 = CA3 ≠ DG. The effects of stress and the stress x region interaction were not significant. PC2 accounts for 18% of the variation and varies significantly between CA1 and CA3 and CA1 and DG (effect of region: $F_{2,15}$= 11.41; $p < 0.001$; Tukey tests: CA1 ≠ DG = CA3). The effects of stress and the stress x region interaction were not significant. PC3 accounts for 15% of the variation and also explains some brain region specific differences (effect of region: $F_{2,15}$= 6.315; $p < 0.01$; Tukey tests: CA1 = DG ≠ CA3), whereas effects of stress and the stress x region interaction were not significant. PC4 is also influences by region ($F_{2,15}$= 6.315; $p = 0.0102$; Tukey tests: CA1 ≠ CA3. PC5 did not account for any significant differences according to region or treatment. PC6 significantly accounted for variance associated with the effect of a stressful experience ($F_{1,16}$> 4.774; p's < 0.04).

The effects of cognitive training on hippocampal transcriptomes

We identified that 423 genes were differentially expressed between the yoked control and cognitively trained animals, 3485 genes that were differentially expressed across subfields, and 324 showed an interaction at FDR $p < 0.05$ (Fig. 3A, B). We see a large effect of brain region on gene expression, with 20% of detectable genes being differentially expressed between one or more brain-region comparisons (3485 differentially expressed genes /17320 measured genes). This is an order of magnitude greater than the 2% of the transcriptome that changed in response to learning (423 DEGs /17320 genes measured).

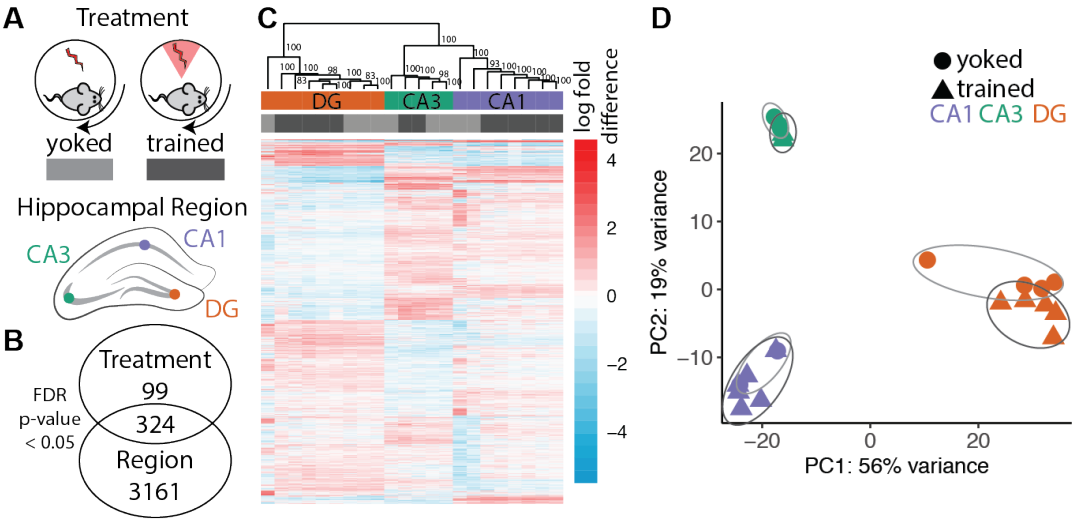

Figure 3. Effects of a learned avoidance behavior on hippocampal transcriptomes. A) Mice used in this study were either subjected to random but mild foot shocks (control) or subjected to mild foot shocks conditioned with spatial cues (trained). Tissue samples were collected from CA1, CA3, and DG. **B)** We identified only 285 genes that were significantly expressed according to the behavioral manipulation and identified 3622 genes that were were differentially expressed between any of the three brain regions. **C)** Hierarchical clustering of the differentially expressed genes gives rise to three distinct clusters corresponding to the three brain regions, with CA1 and CA3 being more similar to one another than to DG. **D)** A principle component analysis of all genes in analysis (regardless of level of significance) shows that PC1 accounts for 50% of the variation and distinguishes the DG samples and the CA1 and CA3 samples (Region: $F_{2,19}$= 199.3; $p = 1.78e-13$). PC2 accounts for 18% of the variation and distinguishes all three subfields ($F_{2,19}$= 220.4; $p = 7.15e-14$). Ellipses were hand-drawn.

Hierarchical clustering of the differentially expressed genes separates samples by both subfield and treatment (Fig. 3C). A principal component analysis of all gene expression data revealed that brain region explains 75% of the variance in the data (Fig. 3D). PC1 accounts for 56% of the variance and distinguishes DG from the Ammon's horn samples (effect of region: $F_{2,19}$= 226.1; $p < 0.001$; Tukey tests: CA1 = CA3 ≠ DG), but the effects of training and the training x

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region interaction were not significant. PC2 accounts for 19% of the variance and distinguishes the three subfields (effect of region: $F_{2,19}= 255.3$; $p < 0.001$; Tukey tests: CA1 ≠ CA3 ≠ DG). PC3 and PC4 indicate a significant influence of cognitive training (PC3: $F_{1,20}=7.451$; $p = 0.01$,) and (PC4: $F_{1,20}=10.11$; $p = 0.005$), but no significant effects of region and the region x treatment interaction.

Identifying unique and general patterns of hippocampal genomic plasticity

Next, we examined the overlap in genomic response to the technical and biological perturbations. We identified three specific genes that responded to both cellular dissociation and to cognitive training: Grin2a, Epha6, and Ltbp3 (Fig. 4A). There was no overlap in differentially expressed genes compared to the cellular dissociation treatment (Fig. 4A).

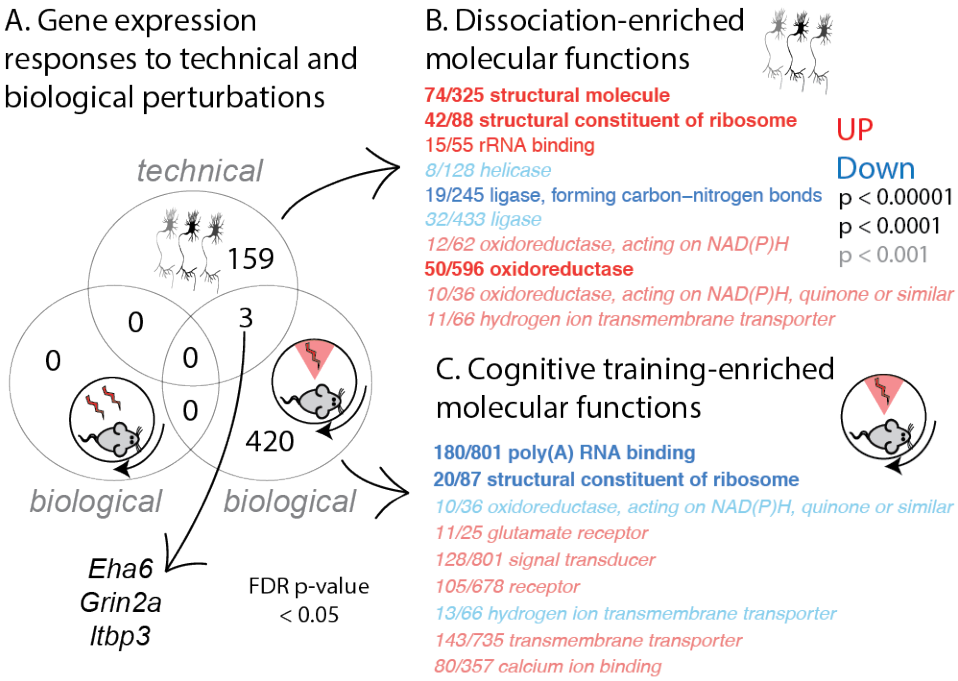

Figure 4. Unique and shared responses to technical treatments and biological perturbations. A) The number of genes that responded to chemical dissociation (163 genes), a stressful experience (0 genes), and cognitive training (423 genes). The three genes that respond to both technical and biological perturbation are Epha6, Grin2a, and Itbp3. **B, C)** The molecular function of gene ontology (GO) categories that are significantly enriches with either up- or down-regulated genes in response to cellular dissociation **(B)** or cognitive training **(C)**. The top 10 most significant GO terms are visualized, each with a p-value < 0.001. The fraction next to GO category name indicates the fraction of genes in that category that survived a 10% FDR threshold for significance. Zero terms survived a 10% FDR threshold in response to a stressful experience.

We next analyzed gene ontology at 5% FDR significant in each of the data sets to identify the molecular function of genes that changed in response to cellular dissociation (Fig. 4B) or cognitive training (Fig. 4C). The process of cellular dissociation results in a significant up-regulation of molecular processes related to ribosomal activity, rRNA binding, oxidoreductase activity, and proton transport, while it caused a down regulation of ligase and helicase activity (Fig. 4B). The GO analysis detected no Molecular Function GO terms in the significantly overrepresented genes in response to the stressful experience. Cognitive training resulted in a significant upregulation of molecular processes related to glutamate receptors, signal transduction, calcium binding, and membrane transport, and it resulted in a significant downregulation of ribosomal activity, oxidoreductase activity, mRNA binding, and proton transport (Fig. 4B). The gene ontology analysis identified 91 significant GO terms in response to cognitive

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training. Among the top 10 are glutamate signaling and membrane transport systems and a downregulation of oxidoreductase and ribosomal activity (Fig. 4C). Notably, learning induces a downregulation of a structural constituent of ribosomes and oxidoreductase, which were both up-regulated in response to cellular dissociation (Fig. 4B,C).

Recovering robust subfield-specific gene expression patterns

Using the public ***Cembrowski et al.*** (***2016b***) dataset, we identified 10,751 genes that were differentially expressed between hippocampal sub-regions (Fig. 5A). Using meta analyses of the public Cembrowski data with the primary data described herein, we identified 146 genes that showed robust subfield-specific gene expression patterns (Fig. 5A). Those 146 genes are enriched in cellular compartments related to synapses and molecular functions related to calcium signaling, GTP exchange, and proteoglycan binding (Fig. 5B).

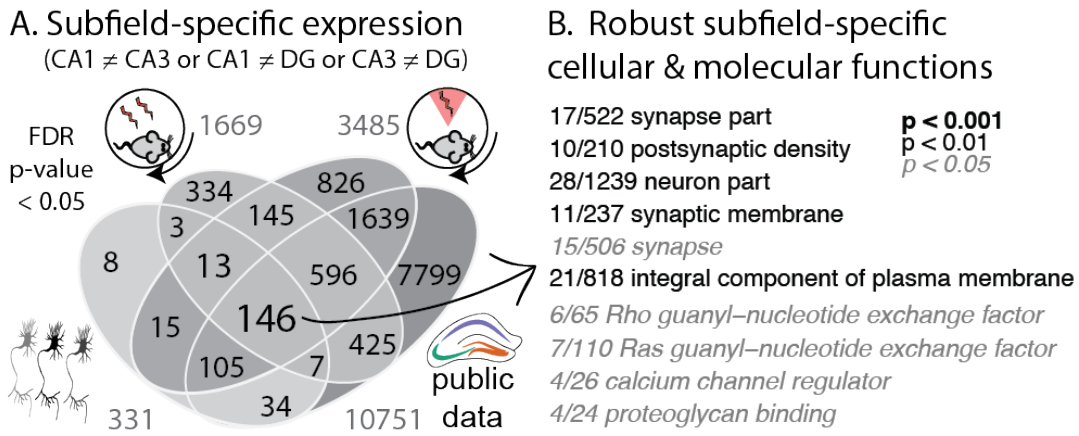

Figure 5. Meta analysis of primary and public data. A) This Venn diagram shows the overlap in brain-region specific gene expression across all four experiments (cellular dissociation, stressor habitation, cognitive training, and a public dataset examining subfield comparisons). Grey numbers indicate total number of differentially expressed genes between and two-way subfield comparison. Using this approach, we identified 146 genes that were differentially expressed between any two subfields of the hippocampus in all four experiments. **B)** Those 146 provide robust brain-region specific markers of gene expression belong to molecular function and cellular compartment GO. The top 10 most significant GO terms are visualized, each with a p-value < 0.05. The fraction next to GO category name indicates the fraction of genes in that category that survived a 10% FDR threshold for significance.

Discussion

The main purposes of this study were 1) to test whether analysis of gene expression in hippocampus subfields is changed by tissue preparation procedures (cellular dissociation versus homogenization) and 2) to evaluate the effects of a stressful experience relative to cognitive training on analysis of gene expression. The work was designed to evaluate the extent to which technical (i.e. cellular dissociation) and biological confounds (i.e. stressful experience) can impact efforts to assess the transcriptomic response to cognitive processes. This is potentially important because it is increasingly necessary to dissociate cells in tissue samples for single cell or single population studies.

Hippocampal subfield differences are well known (***Lein et al., 2004***; ***Hawrylycz et al., 2012***; ***Cembrowski et al., 2016a***,b). Across the three experiments with different treatments, the identity of the hippocampal subfield, explained between 40 and 75% of all the variation in gene expression across samples(Fig. 1D, Fig. 2D, Fig. 3D). The samples that were subjected to cellular dissociation show the least amount of region-specific variation, suggesting that this process might alter the genes that normally distinguish the hippocampal subfields from one another. On the other hand, the ***Cembrowski et al.*** (***2016b***) study identified a larger number

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of genes with subfield specificity; this is likely due to the cell sorting process that generates a relatively homogenous rather than a heterogeneous population of neurons. These results indicate that cell-type specific differences may be recovered by sorting cells into homogenous populations.

Across the data set, many genes consistently or robustly demonstrate hippocampal subfield specificity in their expression (Fig. 5B). The meta-analysis of the primary data and the public data (***Cembrowski et al., 2016b***) identified 146 genes that could potentially serve as robust subfield specific markers. The molecular functions of these potential marker genes are diverse, related to calcium channel regulation, proteoglycan binding, and guanyl-nucleotide (GTP) exchange, as well as cellular compartment categories related to the synapse and the postsynaptic density. This suggests that the phenotypic and functional differences amongst DG, CA3, and CA1 neurons may be driven or influenced by subfield differences in gene expression.

With respect to the effects of cellular dissociation of hippocampal gene expression, we found that 0.9% (162/16,709) of the genes measured changed in response to cellular dissociation (Fig. 1B). This is smaller than the 2.4% (423 /17,320) change we detected in response to cognitive training (Fig. 3B). The stressful experience produces a negligible response (i.e. no significant genes or GO terms were detected), indicating that the mild stress that likely accompanies most behavioral tasks does not have a lasting influence on hippocampal gene expression (Fig. 1B).

The extent to which cellular dissociation and unintended stress impacts the expression of particular genes and signaling pathways, limits the feasibility of investigating how genes contribute to behavior and other responses to organismal manipulations. We found that Grin2a responded to both cellular dissociation and cognitive training (Fig. 4A). Grin2a encodes subunits of N-methyl-D-aspartate (NMDA) type ionotropic glutamate receptors that are crucial for numerous cellular functions throughout the brain, including hippocampus-dependent synaptic plasticity and learning (***Collingridge et al., 1983***; ***Morris, 2013***).Accordingly, care should be taken when studying the role of glutamate and MAPK signaling in combination with cellular dissociation techniques. Epha6 and Ltbp3 also responded to both cellular dissociation and cognitive training (Fig. 4A). Epha6 is involved with the MAPK-Erk signaling pathway. Ltbp3 is involved in binding calcium ions and shows altered gene expression in a mouse model of Alzheimer's Disease (***Neuner et al., 2017***).

We can look beyond the specific genes and examine which pathway responses are concordant or discordant to multiple treatments. In this case, we saw upregulation of ribosomal activity and rRNA biding in response to cellular dissociation, but we saw an opposing downregulation in ribosomal activity and mRNA binding in response to cognitive training (Fig. 5B,C). This suggests that cellular dissociation activates a general transcriptional response whereas cognitive training reduces the transcription of specific protein coding genes. This demonstrates the possibility that such an interaction, in this case a downregulation in response to cognitive training could be overshadowed by technical artifacts if hippocampus tissue is first subjected to the cellular dissociation required for single-cell or single cell population investigations.

We found no detectable transcriptional response in the CA1, CA3, or DG following the stressful experience (Fig. 2B). The shock experience we used causes a large increase in plasma corticosterone levels, comparable to exposure to predator threat, that is observed after the initial shock exposure session but is absent 24-h later after the second training session (Lesburguères et al., 2016b). Our findings support the use of either home cage or shock-yoked animals as controls for active place avoidance training experiments. In the case of the home cage controls that do not experience shock, their stress response is indistinguishable from the trained mice but their sensory experience is very different. In contrast, the shock-yoked mice have identical sensory experience as the experimental mice, but they experience stress that

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the experimental animals do not (***Lesburguères et al., 2016***). It may be that untrained control mice are optimal, because they would have the identical experience of the environment as experimental mice, except at the brief 500 ms moments of shock that account for roughly 3% of the task assuming 20 shocks in 600 s. Depending on the question one control may be preferable over the others, but as demonstrated here, when assessed 24 h after the training experience, they appear to be equivalent in terms of their gene expression profiles (Fig. 2).

Conclusions

Many factors contribute to variation in gene expression. We set out to identify the extent to which the process of cellular dissociation – which allows for single cell analysis of neurons – has an appreciable effect on our ability to detect biologically meaningful variation in hippocampal gene expression. We conclude that there are specific dissociation-induced and cognitive training-induced changes in gene expression that are largely non-overlapping. It is encouraging that the overlap between cellular dissociation and cognitive training is small, indicating that these technical and biological processes affect different transcriptional processes. It is also encouraging to know that the stressful experience had no substantial effect on hippocampal gene expression, which if generalizable to other tasks will allow for using behavioral control groups and behavioral manipulations that also induce modest, potentially confounding stress. These findings provide insight into how cellular and biological manipulations influence gene expression. Through meta analysis and comparison to the published literature, we have identified a subset of robust sub-region specific markers of gene expression profiling. Further research is clearly needed to uncover the influence of other variables on variation in hippocampal gene expression.

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