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Contextual fear conditioning induces differential alternative splicing

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ABSTRACT

The process of memory consolidation requires transcription and translation to form long-term memories. Significant effort has been dedicated to understanding changes in hippocampal gene expression after contextual fear conditioning. However, alternative splicing by differential transcript regulation during this time period has received less attention. Here, we use RNA-seq to determine exon-level changes in expression after contextual fear conditioning and retrieval. Our work reveals that a short variant of Homer1, *Ania-3*, is regulated by contextual fear conditioning. The ribosome biogenesis regulator *Las1l*, small nucleolar RNA *Snord14e*, and the RNA-binding protein *Rbm3* also change specific transcript usage after fear conditioning. The changes in *Ania-3* and *Las1l* are specific to either the new context or the context-shock association, while the changes in *Rbm3* occur after context or shock only. Our analysis revealed novel transcript regulation of previously undetected changes after learning, revealing the importance of high throughput sequencing approaches in the study of gene expression changes after learning.

1. Introduction

Contextual fear conditioning requires two waves of transcription and protein synthesis in the hippocampus to form long-term memory (Bourtchouladze et al., 1998; Igaz, Vianna, Medina, & Izquierdo, 2002). Our lab and others have focused on discovering the genes regulated during these transcriptional waves using both candidate gene and genome-wide approaches. Our microarray-based studies have indicated that the first wave of transcription induces the largest change in gene expression 30 min after contextual learning (Peixoto, Wimmer et al., 2015). However, gene regulation is a complex process that has multiple layers of control. Levels of particular mRNA isoforms can be regulated by alternative start sites, differential splicing including exon skipping and intron retention, and alternative poly(A) site selection (Leff, Rosenfeld, & Evans, 1986; Raj & Blencowe, 2015). Alternative splicing can lead to distinct protein function and interactions (Ellis et al., 2012) or regulate mRNA localization (Ehlers, Fung, O'Brien, & Huganir, 1998; Jaskolski et al., 2004; Papandrikopoulou, Doll, Tucker, Garner, & Matus, 1989), and thus is expected to be particularly important in neurons, which need to traffic mRNA to their long cellular processes.

Most previous research studying genome-wide gene expression in the hippocampus after contextual learning has relied on microarray technology (Barnes, Kirtley, & Thomas, 2012; Cavallaro, D'Agata, Manickam, Dufour, & Alkon, 2002; Keeley et al., 2006; Klur et al., 2009; Levenson et al., 2004; Mei et al., 2005; Peixoto, Wimmer et al., 2015). Although microarrays are a reliable tool to measure changes in gene expression, they are unable to distinguish exon-level effects that are indicative of alternative splicing. RNA-seq provides numerous advantages over

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microarrays (Peixoto, Risso et al., 2015), including the ability to study exon-level changes in gene expression. Isoform-specific gene expression changes are known to occur after fear conditioning, including upregulation of *Bdnf IV*, but not other *Bdnf* isoforms (Lubin, Roth, & Sweatt, 2008; Mizuno, Dempster, Mill, & Giese, 2012), and *Homer1a*, but not *Homer1c* (Mahan et al., 2012) in response to strong, three shock training protocols. The different *Bdnf* isoforms have distinct transcription start sites, while the expression of *Homer1* isoforms is controlled by the splicing regulator SRp20 (Wang, Chikina, Pincas, & Sealfon, 2014), which is upregulated after learning (Antunes-Martins, Mizuno, Irvine, Lepicard, & Giese, 2007). These examples indicate that gene regulation after learning is more complex than gene-level differences and can be highly selective for particular isoforms of a gene.

Therefore, we used RNA-seq to study differential alternative splicing 30 min after contextual fear conditioning and 30 min after memory retrieval. Applying Remove Unwanted Variation (RUV), a recently designed normalization algorithm (Peixoto, Risso et al., 2015; Risso, Ngai, Speed, & Dudoit, 2014), to our data, we discovered 171 bins, corresponding to either an entire exon or any portion of a gene, across 138 genes that showed differential expression after learning independent of changes at the gene-level. After memory retrieval 450 differentially expressed bins corresponding to 311 unique genes were discovered. These bins include retained introns, unique start/end sites, or small RNA not yet spliced out of the polyadenylated mRNA. The differences include *Snord14e*, a small nucleolar RNA, which our lab has previously shown to be regulated at this time point (Peixoto, Wimmer et al., 2015). Sno-RNAs, which are commonly found within introns of genes, regulate RNA processing and have been implicated in memory consolidation (Rogelj, Hartmann, Yeo, Hunt, & Giese, 2003). In addition, *Ania-3*, an alternative short form of Homer1 that has not previously been linked to learning, ribosome biogenesis regulator *Las1l*, and the RNA-binding protein *Rbm3* were also regulated by contextual fear conditioning. These findings demonstrate that alternative splicing is regulated by contextual learning on a genome-wide scale and also identify novel candidate isoforms that may be pertinent to memory consolidation.

2. Materials and methods

2.1. Subjects

C57Bl/6J mice were maintained under standard conditions with food and water available *ad libitum*. Adult male mice 2 months of age were kept on a 12-h light/12-h dark cycle with lights on at 7AM. All behavioral and biochemical experiments were performed during the light cycle with training starting at 10AM (ZT3). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were consistent with National Institutes of Health guidelines.

2.2. Behavior

Contextual fear conditioning was performed as previously described (Hawk et al., 2012; Vecsey et al., 2007) with handling for 3 days prior to conditioning. Briefly, the conditioning protocol entailed a single 2-s, 1.5 mA footshock terminating at 2.5 min after placement of the mouse in the chamber. Mice were left in the chamber for an additional 30 s and then returned to their homecage. One mouse per behavioral group (homecage, fear conditioned) was trained per day over 10 days to reduce unwanted variation caused by training and sacrifice times. One mouse was also tested the next day to ensure proper freezing levels (Peixoto, Wimmer et al., 2015).

2.3. RNA isolation

Hippocampi were dissected either from homecage mice or 30 min after training and placed into RNAlater (Qiagen Valencia, CA) and frozen on dry ice. Tissue was homogenized using a TissueLyser system and RNA was extracted using the RNAeasy Microarray Tissue kit (Qiagen) according to the manufacturer's instructions. Samples were DNase treated using the RNase-Free DNase kit (Qiagen) off-column by incubating 5 μl DNase and 35 μl Buffer RDD for 25 min at RT with each sample. Samples were then ethanol precipitated and resuspended in water.

2.4. RNA-seq library preparation and sequencing

2 μg of RNA from n = 5 homecage and fear conditioned mice was used in the TruSeq RNA Sample Prep Kit (Illumina San Diego, CA) according to the manufacturer's instructions with polyA selection. Completed libraries were size-selected on an agarose gel to remove any high basepair fragments, quantified by qPCR (KAPA Biosystems Boston, MA), and submitted to the PGFI sequencing core at the University of Pennsylvania. An Illumina HiSeq 2000 sequenced the libraries in paired-end 100 bp reads. 3 libraries were sequenced per lane on an Illumina HiSeq 2000, resulting in an average of 67,011,105 reads per sample in the homecage mice and 62,115,805 reads per sample after fear conditioning. Reads had good unique concordance (86.9% in homecage, 85.5% after fear conditioning) and mapping (90.7% of unique concordant reads in homecage and 93.1% after fear conditioning). RNA-seq data is available through GEO (GSE63412) (Peixoto, Risso et al., 2015).

2.5. Data analysis

Sequencing reads were aligned to the mouse mm9 genome using GSNAP (Wu & Nacu, 2010) (http://share.gene.com/gmap). An exon-level count table was produced by counting reads into unique, non-overlapping "bins" using Ensembl gene models and HTSeq (Anders, Reyes, & Huber, 2012) (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html). A "bin" can either be any part of a gene or an entire exon depending on the uniqueness of the region. Bin counts were normalized using upper-quartile scaling implemented in edgeR (Robinson, McCarthy, & Smyth, 2010) followed by RUVs, which corrects for unwanted variation using replicate/negative control samples (Risso et al., 2014). Additionally, we used 8897 bins residing in 625 genes identified as unchanged from a previous microarray experiment as negative controls for RUV under the assumption that these bins are also not changing (Peixoto, Risso et al., 2015; Peixoto, Wimmer et al., 2015). We discovered that four factors of unwanted variation ($k = 4$) need to be adjusted for to resolve the differences caused by contextual fear, which was chosen using the method described by Peixoto, Risso et al. (2015). Differential splicing analysis was performed with the limma Bioconductor package, using the voom and diffSplice functions (Law, Chen, Shi, & Smyth, 2014; Ritchie et al., 2015). Functional annotation was performed through DAVID (Huang da, Sherman, & Lempicki, 2009a, 2009b) (http://david.abcc.ncifcrf.gov/). The annotation was limited to the following sources: GO Biological process, GO Molecular Function, KEGG pathways, and SwissProt and Protein Information Resource keywords and an EASE score restriction of 0.1.

2.6. qPCR analysis

RNA was isolated from a separate cohort of fear conditioned, immediate shock, or context only mice following the same behavioral paradigms described above. Immediate shock consisted of placing the mouse in the context with the footshock on and imme-